SERUM NITRIC OXIDE LEVEL AND ENDOTHELIAL NITRIC OXIDE SYNTHASE GENE INTRON4 VNTR POLYMORPHISM IN ESSENTIAL HYPERTENSION

Dissertation submitted for

M.D. BIOCHEMISTRY BRANCH – XIII
DEGREE EXAMINATION

THE TAMILNADU DR. M.G.R. MEDICAL UNIVERSITY
CHENNAI – 600 032
TAMIL NADU

APRIL 2011

BONAFIDE CERTIFICATE

This is to certify that this dissertation work entitled **SERUM NITRIC OXIDE LEVEL AND ENDOTHELIAL NITRIC OXIDE SYNTHASE GENE INTRON4 VNTR POLYMORPHISM IN ESSENTIAL HYPERTENSION** is the original bonafide work done Dr.G.Sasirekha, Post Graduate Student, Institute of Biochemistry, Madras Medical College, Chennai under our direct supervision and guidance.

Dr.R.Chitraa., M.D., (Guide),
Professor, Institute of Biochemistry,
Madras Medical College,
Chennai – 600 003.

Dr.Pragna B. Dolia., M.D.,
Director and Professor,
Institute of Biochemistry,
Madras Medical College,
Chennai – 600 003.

Dr.J.Mohanasundaram., M.D., PhD., Dip.N.B.,
Dean,
Government General Hospital
Madras Medical College,
Chennai – 600 003.

SPECIAL ACKNOWLEDGEMENT

The author gratefully acknowledges and sincerely thanks Professor **Dr.J. Mohanasundaram M.D.,PhD.,DNB** Dean, Madras Medical College and Government Medical College, Chennai, for granting his permission to utilize the facilities of this Institution for the study.

ACKNOWLEDGEMENT

The author expresses her warmest respects and profound gratitude to **Dr. Pragna B. Dolia, M.D., Director & Professor, Institute of Biochemistry, Madras Medical College** for her able guidance, constant encouragement and support which made this dissertation possible.

The author is extremely thankful to **Dr. R. Chitraa., MD., Professor, Institute of Biochemistry, Madras Medical College** for her constant guidance and encouragement with zeal and enthusiasm during the study.

The author expresses her sincere thanks to **Director & Professor, Institute of Internal Medicine, Dr. C. Rajendiran., M.D.**, for granting permission to draw blood samples for my study from the hypertension clinic.

The author expresses her warm regards to **Professor Dr. K. Ramadevi, Associate Professors Dr. I. Periyandavar, Dr. V. Amudhavalli, Dr. M. Shyamraj, Senior Assistant Professor Dr. V.K. Ramadesikan** and **the Assistant Professors, Dr. S. Sumathi, Dr. Poonguzhali Gopinath, Dr. C.Shanmuga Priya** and **Dr. V. Anandhan of the Institute of Biochemistry, Madras Medical College.**

The author gratefully acknowledges the help rendered by **Mr. A. Venkatesan, Statistician, Department of Evidence Based Medicine, Madras Medical College** for the statistical analysis of the study.

The author expresses her special thanks to all her colleagues for their immense help, constant encouragement and unconditional support throughout the study.

The author is indebted to the patients and the control subjects, from whom blood samples were collected for the study.

Finally, the author expresses her special thanks to her family members especially her beloved parents, for the moral support and encouragement extended by them which gave fulfillment to the dissertation work.

CONTENTS

INTRODUCTION

INTRODUCTION

Essential hypertension is an increase in the systemic arterial blood pressure without an apparent cause. Hypertension affects about 25% of the world population. According to a study conducted in urban areas, the prevalence of systolic and diastolic hypertension in India is 40.9% and 29.3%, respectively (Das,Sanyal, & Basu, 2005). In India, hypertension is the predominant risk factor for coronary artery disease (CAD) in all ethnic groups. Therefore, understanding the pathophysiology of hypertension is important. More than 90% of hypertensive individuals suffer from essential hypertension. It shows an earlier onset in men, than in women. The factors linked to essential hypertension are age, obesity, smoking and stress. A strong genetic predisposition is also suggested. Blood pressure is mainly under the control of blood volume and peripheral resistance, determined predominantly by the arterioles. It is influenced by hormones as well as by the local factors. Nitric oxide (NO), a second messenger of immense importance in the maintenance of blood pressure is a vasodilator, which brings down the peripheral resistance.

NO is synonymous with endothelial derived relaxing factor (Katzung, 2004). It acts via the heme moiety of guanylyl cyclase to produce cyclic guanosine mono-phosphate (cGMP). This reduces the levels of cytosolic Ca^{++} and also phosphorylates myosin light chain kinase(MLCK). NO decreases the activity of platelets and neutralizes free radicals. Thus, it plays a major role in preventing atherosclerosis. It prevents binding of leucocytes to the endothelium and decreases inflammation. Hence, it is considered as an important factor in preventing hypertension. There is a close link between the level of available NO and blood

pressure. Various studies suggest that, the factors influencing the levels of NO will have an important role in the pathophysiology and susceptibility to essential hypertension.

The main source of circulating NO is the endothelium, where it is produced from the amino acid L-arginine by the action of endothelial nitric oxide synthase (eNOS). This is a constitutive enzyme with its gene (eNOS/*NOS3*) on chromosome 7. NOS is a heme protein that exists in its inactive form as a monomer, but dimerizes before action. NO is produced by the neurons too, where neuronal nitric oxide synthase (nNOS/ *NOS1*), another constitutive enzyme, is responsible for its production. Inducible nitric oxide synthase (*iNOS/NOS2*) on chromosome 17 is induced mostly during inflammation. It is responsible for the harmful effects of the gas. It is expressed in macrophages, smooth muscle cells and hepatocytes and is responsible for pathological vaso-relaxation. The eNOS is membrane bound, while the other two are present in soluble form. NO is removed from circulation mostly by reaction with free radicals, such as superoxide. The balance between the production and removal of NO is very important with regard to hypertension .

Because eNOS is the major enzyme responsible for nitric oxide production, variation in its expression and activity can be linked to hypertension. It has been found that, a G to T polymorphism in the exon 7 region leading to a change from glutamate at 298 position to aspartate decreases the expression of the enzyme, but has no effect on the activity (Kato et al., 1999). Another study has suggested that, such a change causes the enzyme to undergo selective proteolysis (Hingorani, 2003).

In intron 4 of *NOS3,* there can be four 27 bp repeats (allele ‘a’) or five (allele ‘b’). Presence of allele ‘a’, rather than the wild-type allele ‘b’, though shows an increase in the expression of the enzyme, reduces its activity (Kato et al. 1999). The promoter region T to C polymorphism has also been shown to reduce the expression of the enzyme. The association of these polymorphisms to hypertension has been controversial. Whereas, the G to T polymorphism has been associated with hypertension in many ethnic groups (Miyamoto et al., 1998), including the Indian population (Srivastava K, Narang R, Sreenivas V, Das S and Das N ,2008), the association of the other two polymorphisms has been more controversial (Hingorani, 2003; Kato et al., 1999). Few studies have been conducted on Asians, especially those living in India, regarding these polymorphisms.

Hence, it is proposed to study the association of serum NO level and eNOS intron4 polymorphism in essential hypertension.

REVIEW OF LITERATURE

SYSTEMIC HYPERTENSION

Affecting 1 billion people worldwide, **systemic hypertension** remains the most common, readily identifiable and reversible risk factor for myocardial infarction, stroke, heart failure, atrial fibrillation, aortic dissection and peripheral arterial disease. Due to the escalating obesity and population aging in developed and developing countries, the global burden of hypertension is rising and projected to affect 1.5 billion persons, one third of the world's population, by the year 2025. Thus, hypertension one of the world's great public health problems [1] remains as the leading cause of death, worldwide .

EPIDEMIOLOGY :

The prevalence of hypertension has been increasing, with 972 million people suffering from it .

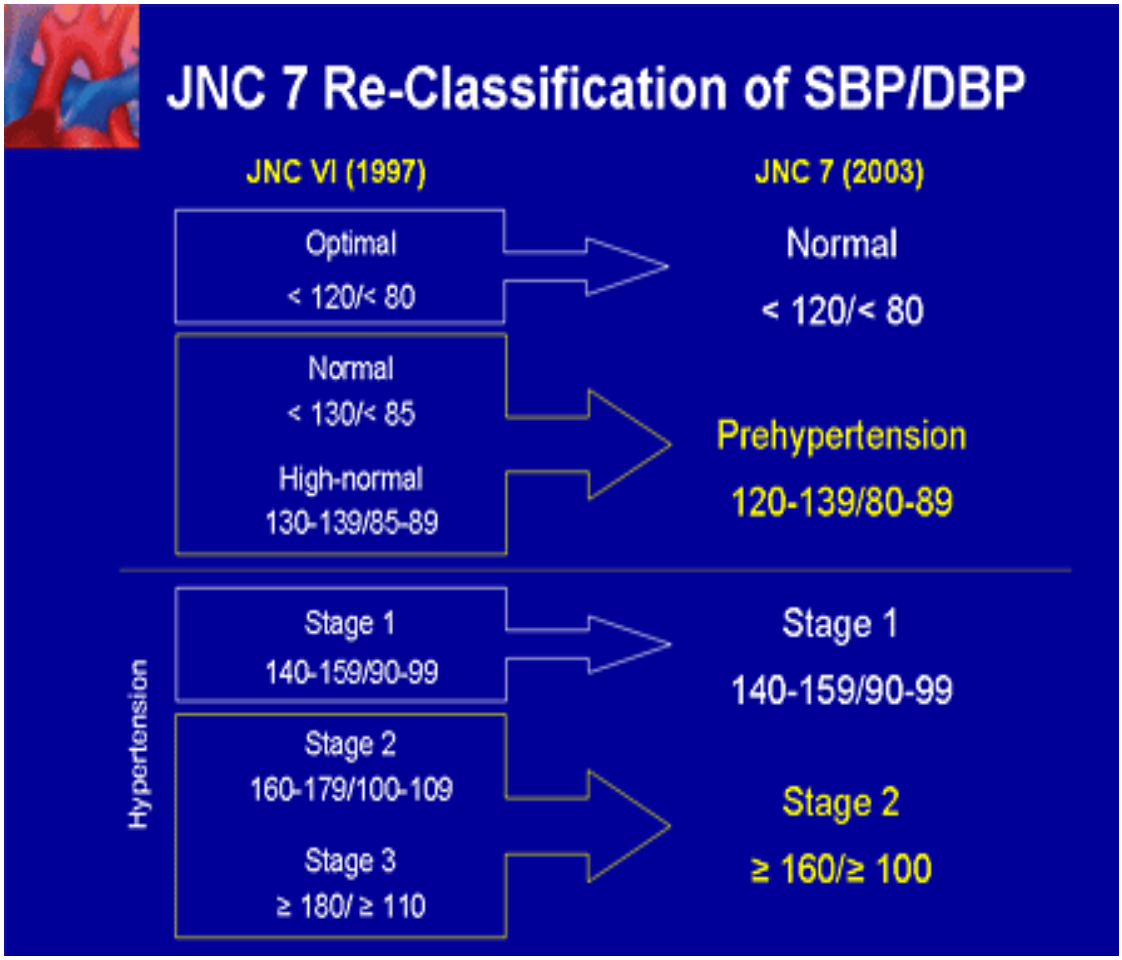
- Incidence rate of hypertension ranges between 3% and 18% , depending on the age , gender , ethinic groups and body size of the population studied. [25]

- Age and sex adjusted increase in the prevalence of hypertension from 153.1 per 1000 adults in 1995 to 244.8 per 1000 in 2005, shows a relative increase of 60 % .

- Age and sex adjusted increase in the incidence of hypertension from 25.5 per 1000 adults in 1997 to 32.1 per 1000 in 2004 was a relative increase of 25.7 % .

Figure : 1

CLASSIFICATION OF SYSTEMIC HYPERTENSION – JNC 7



- Researchers have predicted a relative increase of 24% in the prevalence of hypertension in developed countries from 2000 to 2025.[4]

DEFINITION AND CLASSIFICATION OF HYPERTENSION:

Currently, hypertension is defined as an usual blood pressure of 140/90 mm Hg or higher levels, for which the benefits of pharmacological treatment have been definitively established in randomized placebo-controlled trials[4] (Figure : 1)

In 90 to 95 % of hypertensive patients, a single reversible cause of the elevated blood pressure cannot be identified—hence, the term **PRIMARY HYPERTENSION**. In the remaining 5 to 10 % , a more discrete mechanism can be identified, and the condition is termed **SECONDARY OR IDENTIFIABLE HYPERTENSION.**

GENE ENVIRONMENTAL INTERACTION : Hypertension is a multi-factorial disease, involving both environmental and genetic components.[1] (Figure:2)

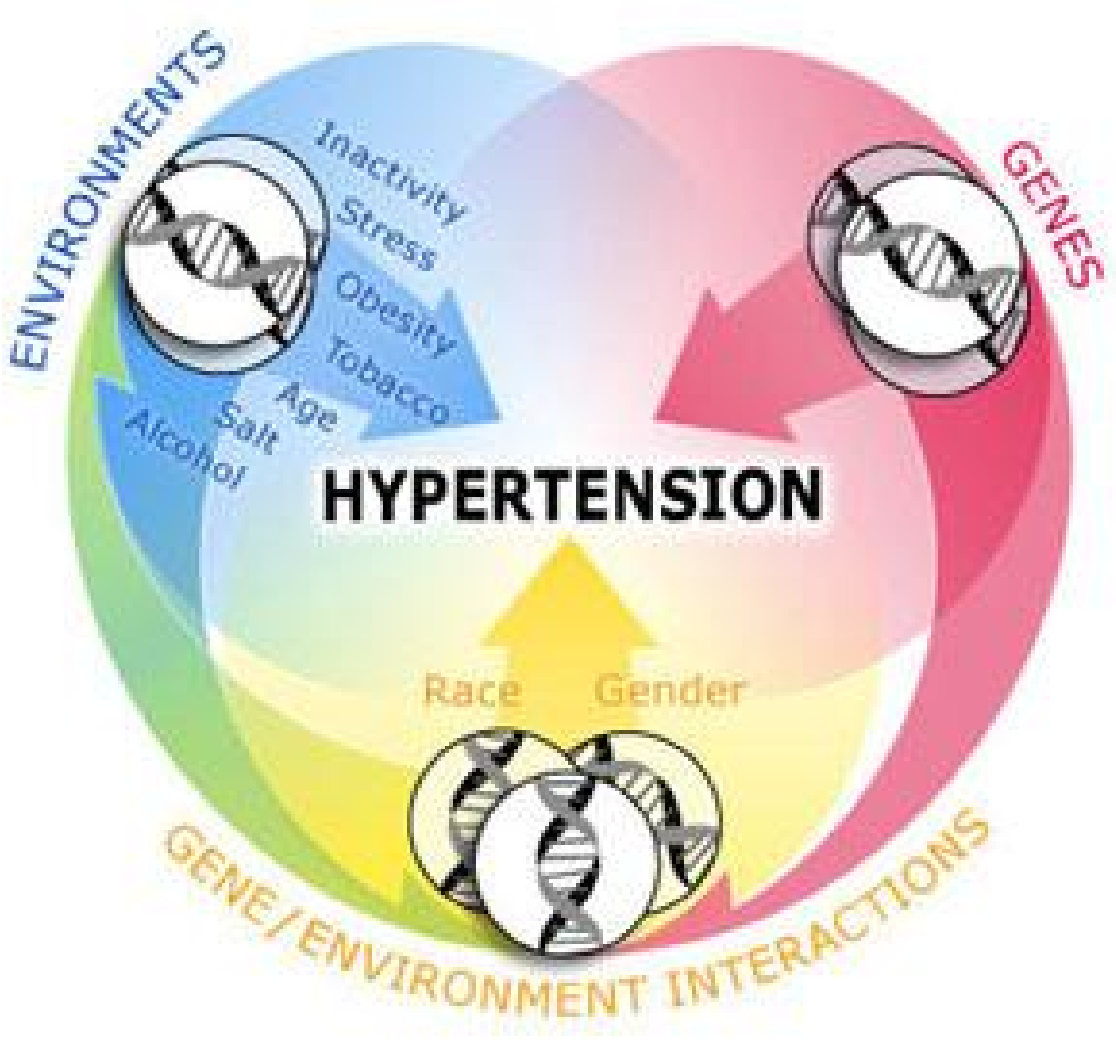
ENVIRONMENTAL AND BEHAVIORAL DETERMINANTS[2] :

In most patients with primary hypertension, readily identifiable behaviors contribute to the elevated blood pressure.

1. The nicotine in cigarette smoke transiently raises blood pressure by 10 to 20 mm Hg with every single cigarette, thereby elevating the average daytime blood pressure in habitual smokers.

Figure : 2

GENE ENVIRONMENT INTERACTION IN CAUSATION OF ESSENTIAL HYPERTENSION



2. With alcohol, the risk of hypertension is lower in moderate drinkers (one or two drinks per day) than in teetotalers but increased in heavy drinkers (three or more drinks per day).

3. Caffeine consumption typically causes only a small transient rise in blood pressure which, in some individuals, habituates after the first cup of coffee. Caffeine consumption does not increase the risk of developing hypertension.[5]

4. In normotensive individuals, habitual physical inactivity associates with a markedly increased risk of developing hypertension to certain extent , due to weight gain.

5. Lifetime dietary habits greatly influence the risk of developing hypertension. Diets low in fresh fruits, may be associated with an increased risk of developing hypertension, perhaps because of lower citrate intake.[6]

6. The two most important dietary determinants of hypertension, however, are consumption of excessive calories and salt.

7. Across various populations, hypertension prevalence increases linearly with average body mass index. With the unrelenting obesity epidemic, more than 50% of all cases of hypertension can be attributed to obesity.

8. The risk of developing hypertension is linearly and tightly related to dietary sodium intake.[7] Inter-individual variability in blood pressure, responses to dietary sodium loading and sodium restriction, indicates an important genetic underpinning.

GENETIC DETERMINANTS :

There are many evidences to show that genetic factors contribute to blood pressure regulation and hypertension.

1. The normal distribution of blood pressure in the general population indicates the presence of multiple environmental and genetic factors ; thus, a polygenic etiology.

2. Rare monogenic forms of hypertension associated with major defects in salt handling by the renal system, prove that gene mutations can cause hypertension, leading to a hypothesis that minor variations in these genes may contribute to essential hypertension.

3. From a population perspective, there is a considerable evidence to indicate the presence of a heritable component from family aggregation studies. Hypertension is more common in individuals who have one or two hypertensive parents and is closely correlated in mono-zygotic than di-zygotic twins [24,25] .

It is estimated that, around 30% of variation in blood pressure is due to genetic factors [23].

In cases of secondary hypertension, it is well proved that genetic mutations are involved. Fourteen genes have been identified that cause Mendelian forms of hypertension.[8]Some of the examples are

- Liddle's syndrome is a disorder associated with hypertension, low plasma renin and aldosterone levels with hypokalemia, responding to amiloride, an inhibitor of the distal renal epithelial sodium channel.

- Glucocorticoid-remediable aldosteronism is a disorder mimicking Conn's syndrome, with a chimeric gene formed from portions of the 11β-hydroxylase gene and the aldosterone synthase gene. This defect resulting in hyper-aldosteronism , responding to dexamethasone, presents with a high incidence of stroke.

- Congenital adrenal hyperplasia due to 11β-hydroxylase deficiency is a disorder that has been associated with 10 different mutations of the CYP11B1 gene.

- Syndrome of apparent mineralocorticoid excess, due to mutations in the gene encoding the renal enzyme 11α-hydroxysteroid dehydrogenase, allows normal circulating concentrations of cortisol to activate the mineralocorticoid receptors.

- Congenital adrenal hyperplasia with 17α-hydroxylase deficiency, is a disorder with hypo-reninemia, hypoaldosteronism due to hypokalemia (due to urinary potassium wasting) and absence of secondary sexual characteristics.

- Gordon's syndrome (pseudo-hypoaldosteronism) is associated with familial hypertension and hyperkalaemia, possibly related to the long arm of chromosome 17.

- Sporadic case reports show familial inheritance of phaeochromocytoma (multiple endocrine neoplasia, MEN-II syndrome), Cushing's syndrome, Conn's syndrome and renal artery stenosis due to fibromuscular dysplasia.

  Various studies are being conducted to explore the genes involved in essential hypertension :

- The angiotensinogen gene may be related to hypertension. Whereas, angiotensin converting enzyme gene may be related to left ventricular hypertrophy or hypertensive nephropathy.

- α-adducin gene mutation may be associated with salt sensitive hypertension. This presents with sodium retention due to a faster transport across the Na, K-ATPase pump.

- **eNOS gene polymorphism may be associated with hypertension , by influencing serum NO level.**

HEMODYNAMIC SUBTYPES OF ESSENTIAL HYPERTENSION :

Hypertension can be divided into three distinct hemodynamic subtypes, that vary sharply by age.[2]

DIASTOLIC HYPERTENSION IN MIDDLE AGE :

When hypertension is diagnosed in middle age (typically from 30 to 50 years of age), the most common blood pressure pattern is, elevated diastolic pressure with systolic pressure being normal (*isolated diastolic hypertension*) or elevated (*combined systolic-diastolic hypertension*). This is classic **"ESSENTIAL**

HYPERTENSION." Isolated diastolic hypertension is more common in men and is often associated with middle-age weight gain.[9] Without treatment, isolated diastolic hypertension often progresses to combined systolic-diastolic hypertension. The fundamental hemodynamic fault is an elevated systemic vascular resistance coupled with an inappropriately normal cardiac output. Vasoconstriction at the level of the resistance arterioles, results from an increased neuro-hormonal drive and an autoregulatory reaction of vascular smooth muscle to an expanded plasma volume, the latter is due to impairment in the renal ability to excrete sodium.

ISOLATED SYSTOLIC HYPERTENSION IN OLDER ADULTS :

After 60 years, isolated systolic hypertension (systolic blood pressure above 140 mm Hg and diastolic blood pressure below 90 mm Hg) is the most common form[10]. Isolated systolic hypertension may represent an exaggeration of this age-dependent stiffening process compared with young or middle-aged adults with optimal blood pressure. Those with blood pressure in the high-normal range (borderline or pre-hypertension) are much more likely to develop isolated systolic hypertension after 60 years[9].

ISOLATED SYSTOLIC HYPERTENSION IN YOUNG ADULTS :

At the other end of the age spectrum is isolated systolic hypertension in young adults who are typically 17 to 25 years of age. The key hemodynamic abnormalities are, increased cardiac output and a stiff aorta, both presumably reflecting an overactive sympathetic nervous system[11].

MECHANISMS OF PRIMARY (ESSENTIAL) HYPERTENSION :

A number of physiological mechanisms are involved in the maintenance of normal blood pressure and, their derangement may play a role in the development of essential hypertension.

Many inter-related factors contribute to the raised blood pressure in hypertensive patients and their relative roles may differ between individuals. The factors that have been intensively studied are salt intake, obesity / insulin resistance, the renin-angiotensin system and the sympathetic nervous system. In the past few years, other factors have been evaluated including genetics, endothelial dysfunction (as manifested by changes in endothelin and nitric oxide), low birth weight & intrauterine nutrition and neurovascular anomalies.

1. NEURAL MECHANISMS :

In young adults, primary hypertension consistently is associated with increased heart rate and cardiac output, increased plasma and urinary norepinephrine levels, regional norepinephrine spillover, peripheral postganglionic sympathetic nerve firing (by microelectrode recordings) and alpha-adrenergic receptor–mediated vasoconstrictor tone in the peripheral circulation[12,13]. Central sympathetic outflow can be driven by deactivation of inhibitory neural inputs (e.g., baroreceptors), activation of excitatory neural inputs (e.g., carotid body chemoreceptors, renal afferents) or by circulating angiotensin II , which activates pools of excitatory brain stem neurons, that are devoid of a blood-brain barrier.

BARORECEPTORS AND HYPERTENSION :

In hypertension, the baroreceptors are reset to defend a higher level of blood pressure. Baro-reflex control of sinus node function is impaired even in mild hypertension, but baroreflex control of systemic vascular resistance and blood pressure is well preserved. Partial baroreceptor dysfunction is common in elderly hypertensives and typically presents with a triad of orthostatic hypotension, supine hypertension and symptomatic postprandial hypotension, the latter initiated by splanchnic pooling after carbohydrate-rich meals[14].

OBESITY-RELATED HYPERTENSION:

Neural mechanisms of obesity-related hypertension deserve special mention. With weight gain, reflex sympathetic activation is thought to be an important compensation to burn fat but, at the expense of sympathetic over-activity in target tissues (i.e., vascular smooth muscle and kidney) that produce hypertension[15]. Near-maximal rates of sympathetic firing are seen in hypertensive patients with the metabolic syndrome, with or without “new-onset type 2 diabetes”. Although the sympathetic activation associates with insulin resistance, the precise stimulus to sympathetic outflow is unknown (leptin, other adipokines, and angiotensin-II being potential candidates).

SLEEP APNEA AS A CAUSE OF NEUROGENIC HYPERTENSION :

The strongest evidence for sustained neurogenic hypertension in humans is, in patients with obstructive sleep apnea. With repeated arterial de-saturation during apneas, activation of carotid body chemoreceptors not only causes dramatic pressor

episodes throughout the night but, also resets the chemoreceptor reflex. Daytime normoxia is misinterpreted as hypoxia, producing sustained reflex sympathetic activation and hypertension, even during waking hours[16]. Obstructive sleep apnea not only predisposes to hypertension, but also accelerates the risk of several hypertensive complications (e.g., stroke, atrial fibrillation and cardiovascular death) beyond that explained by blood pressure elevation alone[17].

2. RENAL MECHANISMS :

The kidney is the culprit as well as the victim in hypertension, producing a vicious cycle of progressive renal dysfunction and hypertension. In many forms of experimental and human hypertension, the fundamental abnormality is an acquired or inherited defect in the renal ability to excrete the excessive sodium load imposed by a high sodium chloride diet. Renal sodium retention expands the plasma volume, increasing cardiac output and triggering auto-regulatory responses that increase systemic vascular resistance. Salt retention augments the smooth muscle contraction produced by all known endogenous vasoconstrictor substances.

LOW BIRTH WEIGHT :

Low birth weight with reduced nephrogenesis, increases the risk of developing adult salt-sensitive hypertension. Adult hypertensives having fewer glomeruli per kidney but very few obsolescent glomeruli, suggest that nephron drop-out and decreased total filtration surface area is the cause, and not the consequence of the hypertension.[18]

GENETIC CONTRIBUTIONS :

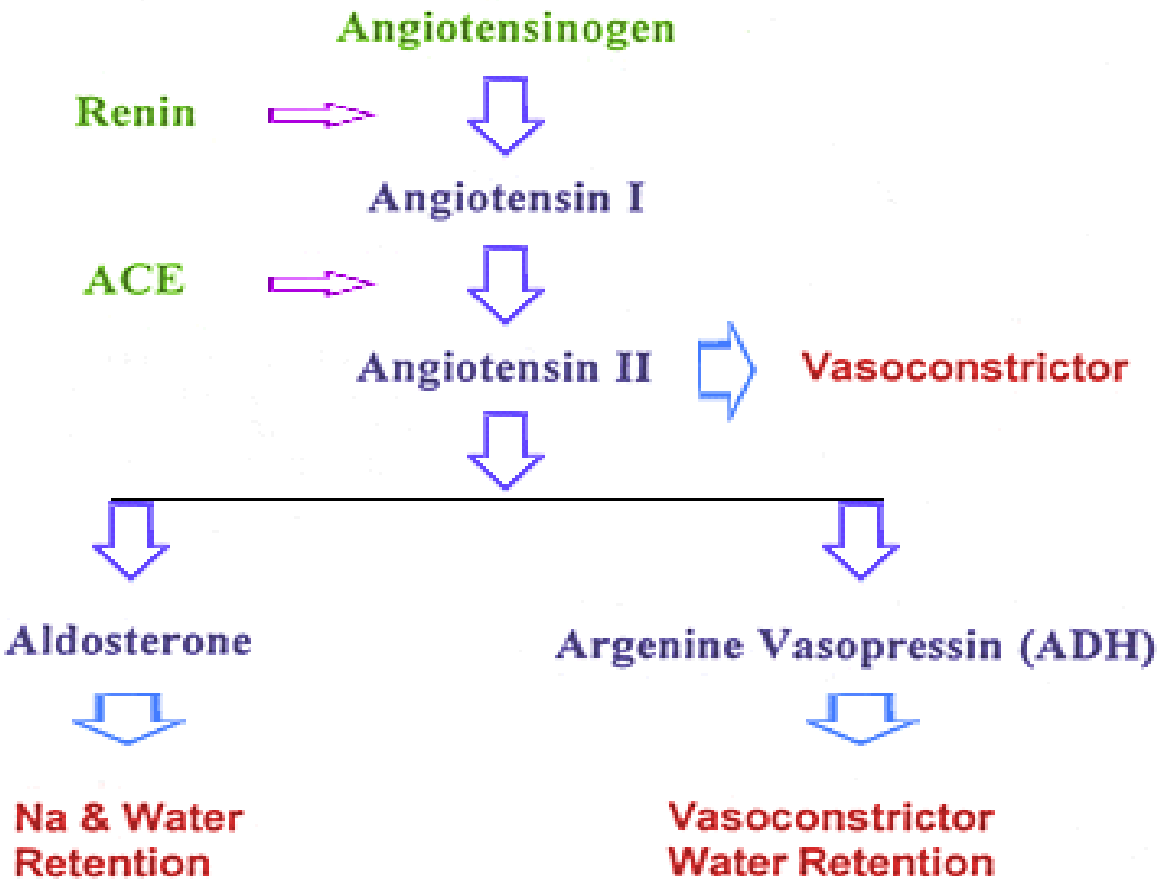
Animal and human studies have implicated an important genetic contribution to salt-sensitive hypertension. Rats with in-bred defects in the kidneys' ability to excrete sodium, remain relatively normotensive on a sodium-restricted diet but become severely hypertensive when fed with a high-sodium diet, a model of salt-sensitive hypertension that can be cured by renal transplantation.

3. **HORMONAL MECHANISMS: RENIN-ANGIOTENSIN-ALDOSTERONE SYSTEM (Figure : 3) :**

Activation of the renin-angiotensin-aldosterone system (RAAS) is one of the most important mechanisms contributing to endothelial cell dysfunction, vascular re-modeling, and hypertension . Renin, a protease produced solely by the renal juxtaglomerular cells, cleaves angiotensinogen (renin substrate produced by the liver) to angiotensin-I, which is converted by angiotensin-converting enzyme (ACE) to angiotensin-II . ACE is most abundant in the lungs but is also present in the heart and systemic vasculature (tissue ACE). Chymase, a serine protease in the heart and systemic arteries, provides an alternative pathway for conversion of angiotensin-I to angiotensin-II. The interaction of angiotensin-II with G protein–coupled angiotensin-1 receptors activates numerous cellular processes that contribute to hypertension and accelerate hypertensive end-organ damage . These include vasoconstriction, generation of reactive oxygen species, vascular inflammation, vascular and cardiac remodeling and production of aldosterone, the principal mineralocorticoid. There is increasing evidence that aldosterone, angiotensin-II, and

Figure : 3

RENIN ANGIOTENSIN ALDOSTERONE SYSTEM



even renin and pro-renin activate multiple signaling pathways, that can damage vascular health and cause hypertension.

ALDOSTERONE AND EPITHELIAL SODIUM CHANNEL REGULATION :

RAAS activation is a major homeostatic mechanism to counter hypovolemic hypotension (as with hemorrhage or salt and water deprivation). Interaction of aldosterone with cytosolic mineralocorticoid receptors in the renal collecting duct cells, recruits sodium channels from the cytosol to the surface of the renal epithelium. The epithelial sodium channels (eNaCs) so recruited increase sodium re-absorption, thereby re-expanding plasma volume.

So, in the setting of high dietary sodium and elevated blood pressure, the RAAS should be completely suppressed, and any degree of RAAS activity is inappropriate. In normotensive individuals, the risk of developing hypertension increases with increasing levels of serum aldosterone, that are well within the normal range.[20] In African Caribbean hypertensives, serum aldosterone levels are higher than in white hypertensives, despite lower plasma renin levels,[21] implicating abnormal aldosterone production by renin-independent mechanisms, a form of primary aldosteronism. By stimulating mineralocorticoid receptors in the heart and kidney, circulating aldosterone may contribute to the development of cardiac and renal fibrosis in hypertension[22]. By stimulating mineralocorticoid receptors in the brain stem, aldosterone also may contribute to sympathetic overactivity.

RECEPTOR-MEDIATED ACTIONS OF ANGIOTENSIN II :

Two main types of angiotensin receptors are known. Angiotensin-1 receptors are widely expressed in the vasculature, kidney, adrenals, heart, liver and brain. Angiotensin-1 receptor activation explains most of the hypertensive actions of angiotensin-II. Furthermore, enhanced angiotensin-1 receptor mediated signaling, provides a central mechanistic explanation for the frequent coexistence of elevated blood pressure with insulin resistance and atherosclerosis. Angiotensin 2 receptors are widely distributed in the fetus but, in adults, are found only in the adrenal medulla, uterus, ovary, vascular endothelium and distinct brain regions.

4. VASCULAR MECHANISMS:

Alterations in the structure and function of small and large arteries play a pivotal role in the pathogenesis and progression of hypertension. The endothelial lining of blood vessels is critical to vascular health and constitutes a major defense against hypertension.

Various studies have been carried out which had revealed the importance of endothelium in regulating vascular function :

- In 1977, Moncada et al published the first report indicating that, the endothelium plays a central role in the control of vascular tone via the production of vasoactive substances [26].
- In 1980, Furchgott and Zawadzki [27] demonstrated in an experimental preparation of the rabbit aorta, the obligatory role played by endothelial cells

in vascular relaxation in response to effectors like acetylcholine and postulated the existence of a vascular relaxing factor, derived from the endothelium.

- In 1987, two research groups, lead by Ignarro et al [28] and by Palmer et al [29] demonstrated that the relaxing factor derived from the endothelium was **NO** an odorless gas until then, considered as a mere pollutant.

- Endothelial dysfunction was first characterized in humans in 1986 by Ludmer et al[30] , who demonstrated that atherosclerotic coronary arteries contracted in response to intracoronary infusion of acetylcholine, while normal coronaries showed dilatation.

- In 1992, endothelial dysfunction was documented by Celermajer et al [31] in children and otherwise healthy young adults with risk factors for atherosclerosis.

- Recent clinical studies have demonstrated that, some drugs well known to reduce the incidence of cardiovascular events, improve endothelial function [43-46].

THE ENDOTHELIUM IN CARDIOVASCULAR HOMEOSTASIS :

Vascular endothelium may be considered a dynamic, heterogeneous organ, having secretory, synthesizing, metabolic and immunological functions, vital to human beings [47]. The endothelium regulates the flow of nutrient substances of various biologically active molecules and of blood cells through the entire human

body. It is selectively permeable, possessing various cell membrane receptors for molecules that include proteins (growth factors, coagulation, and anticoagulation proteins), lipid-transporting particles (LDL), metabolites (nitric oxide, serotonin) and hormones (endothelin-1). Under physiological conditions, the endothelium keeps a reduced vasomotor tone, prevents leukocyte /platelet adhesion and inhibits the proliferation of vascular smooth muscle cells .

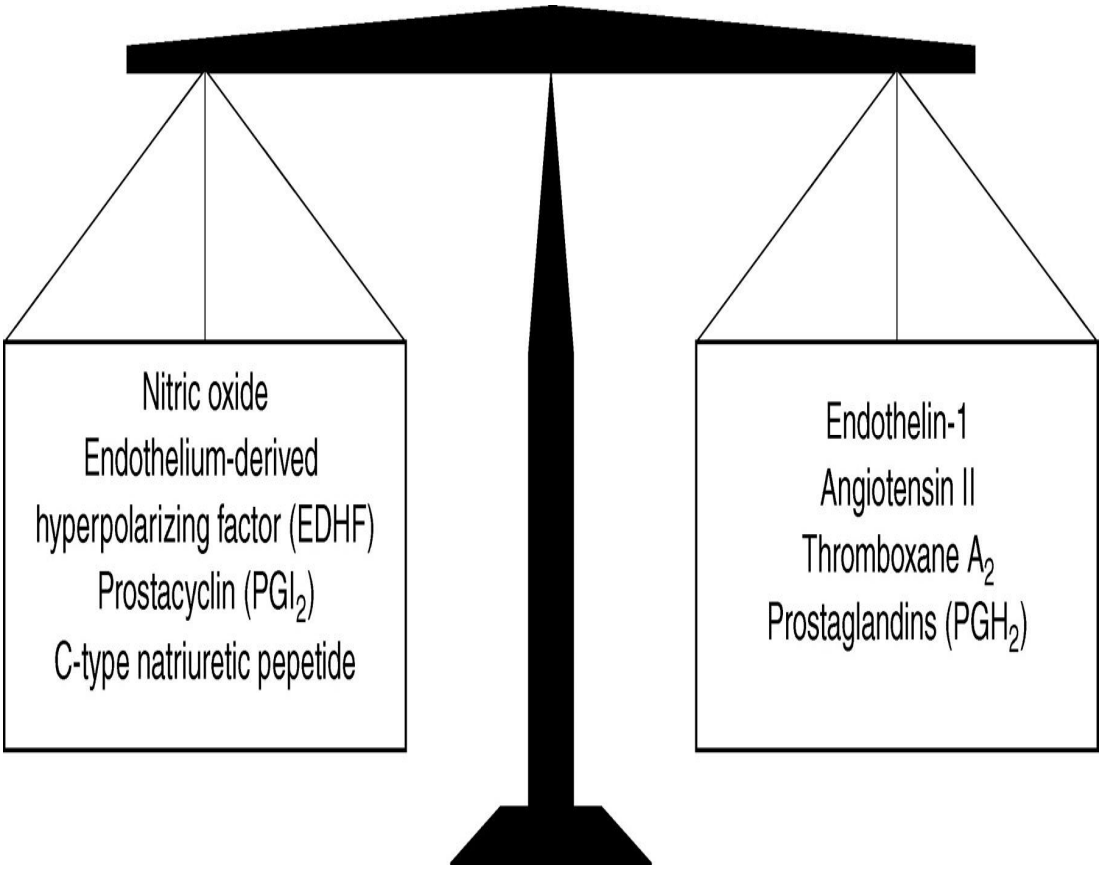
The endothelium possesses important anticoagulant, anti-platelet and fibrinolytic actions. Endothelial cells are the largest sites of reactions involving thrombin [48]. Some of the stimuli that activate platelets (adenosine diphosphate and thrombin), stimulate the release of prostacyclin by the endothelium, inhibiting platelet aggregation [26,49]. In response to stimuli like nor-adrenaline, vasopressin, thrombin and vascular stasis, endothelial cells secrete tissue plasminogen activator [50], a potent thrombolytic agent with wide clinical application, thus providing a defense against uncontrolled coagulation. Other hemostatic factors secreted by the endothelium, include plasminogen activating factor (PAI-1) inhibitor, Von Willebrand factor and thrombomodulin. When stimulated by certain physical or chemical factors, the endothelial cell undergoes phenotypical modifications that determine its transformation into a thrombogenic surface. The dynamic equilibrium existing between these two states often permits the endothelial cell to return to its basal state, once the thrombogenic stimulus has ceased.

ENDOTHELIAL VASOMOTOR FUNCTION : (Figure : 4)

The endothelium plays a central role in the regulation of vascular tone and blood flow, by the secretion and capture of paracrine vasoactive substances, which

Figure 4 :

ROLE OF ENDOTHELIUM IN REGULATING BLOOD PRESSURE



includes vasodilator substances (NO, prostacyclin & endothelium-derived hyperpolarizing factor) and vasoconstrictor substances (endothelin-1 , thrombaxane A2 & platelet-activating factor).

VASODILATORS :

NO is the main mediator of vasomotor tone regulation in physiological situations, small amounts being continuously secreted by the endothelial cells [29,51] to maintain a reduced arterial tone in the systemic and pulmonary circulation [52]. The vasodilator activity of NO is due to its interaction with the iron atom of the heme prosthetic group of guanylyl cyclase, causing its activation and increasing the intracellular levels of cyclic guanosine monophosphate (cGMP) [53]. In smooth muscle cells, this decreases intracellular calcium concentration, causing vascular relaxation[54].

Prostacyclin is synthesized from arachidonic acid by cyclo-oxygenase [26], being rapidly produced and released from endothelial cells[55], in response to humoral and hemodynamic factors. It interacts synergistically with nitric oxide, causing vasodilation & inhibition of platelet adhesion and aggregation [56]. The stimulation of adenylyl cyclase and increased intracellular concentration of cAMP in smooth muscle cells and platelets mediate its actions. Prostacyclin does not appear to be continuously produced by endothelial cells [57], but to be synthesized in response to specific stimuli like bradykinin, adenosine diphosphate, hypoxia, and shear stress.

Endothelium-derived hyperpolarizing factor, another vasodilator substance produced by the endothelium, promotes vascular smooth muscle cell

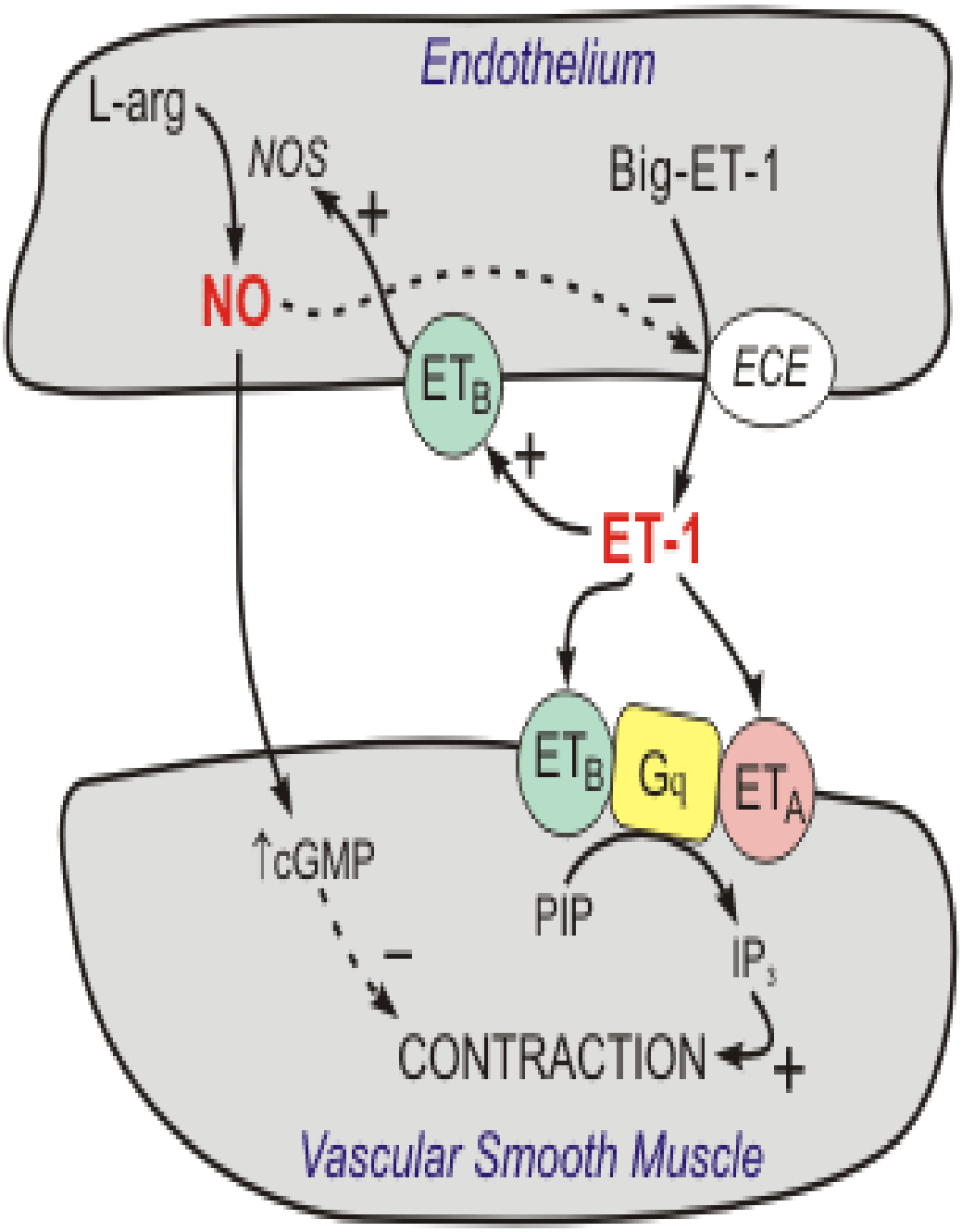
relaxation by increasing cell membrane conductance of potassium [58]. This factor is also secreted in response to acetylcholine and blocked by Ouabain, an inhibitor of Na/K ATPase. The endothelium-derived hyperpolarizing factor has not yet been isolated and its physiological role remains uncertain.

VASOCONSTRICTORS :

. **Endothelins**[26] (Figure 5) constitute a family of polypeptides produced by various cell types. Of the three isoforms known, endothelial cells appear to produce only endothelin-1. This is a 21 amino-acid peptide formed from its inactive precursor pre-endothelin-1, by endothelin converting enzyme. It plays its role as an arterial blood flow regulator in both normal and pathologic conditions [59]. Endothelin-1 formation and release are stimulated by angiotensin II, anti-diuretic hormone, thrombin, cytokines, reactive oxygen species, hypoxia and shearing forces acting on the vascular endothelium. Endothelin -1 release is inhibited by prostacyclin and atrial natriuretic peptide as well as by NO. Binding to specific receptors in vascular smooth muscle cells it increases intracellular concentration of calcium, leading to vasoconstriction[60] . Intra-myocardial vessels are more sensitive to endothelin, suggesting that this peptide plays a major role in control of blood flow. It is interesting that, in functionally intact endothelia, endothelin stimulates the production of NO and of prostacyclin, which, therefore, modulates vasoconstrictor action and reduces the synthesis of endothelin itself. Two types of vascular receptors for endothelin have been identified. Receptor endothelin B is observed in endothelial cells, being responsible for the stimulation of NO and prostacyclin formation. Both the endothelin receptors, observed in smooth muscle cells, mediate contraction and

Figure : 5

ENDOTHELIN RECEPTORS AND ITS INTERACTION WITH NITRIC OXIDE



proliferation of these cells. A large number of endothelin receptor antagonists developed in recent years, are being tested experimentally and clinically.

Thromboxane A2 and **prostaglandin H2** are constrictor factors also secreted by the endothelium. They activate the thromboxane receptor in smooth muscle cells and platelets, in opposition to the effects of NO and prostacyclin. However, the role of these substances in coronary circulation has not been clearly established. **Platelet activation factor** is another vasoconstrictor synthesized and released by endothelial cells in response to humoral and hemodynamic stimuli, which probably participates in the regulation of vasomotor tone.

Finally, the endothelium expresses the **ACE**, which metabolizes bradykinin. In addition, angiotensin II directly stimulates the production of endothelin.

PATHOPHYSIOLOGY OF ENDOTHELIAL DYSFUNCTION :

Endothelial dysfunction is characterized by the reduction of the endothelium-derived vasodilators, by local increases in antagonists to these substances or by an association of these two factors . Endothelial dysfunction appears to play a pathogenic role in the initial development of atherosclerosis[32-34] and of unstable coronary syndromes[35], and their diverse risk factors viz hypercholesterolemia[36], smoking[37], hypertension [38], diabetes mellitus [39], family history of premature coronary disease [40], hyperhomocysteinemia [41] and aging [42].

NO and endothelin are the two main factors involved in endothelial dysfunction. High plasma concentrations of **endothelin-1** have been reported in myocardial infarction, cardiogenic shock, unstable angina pectoris, coronary artery

disease in general, cardiac failure and essential hypertension [73,74]. Endothelin-1 action, unopposed by NO, tends to promote vasoconstriction and proliferation of vascular smooth muscle cells in states of endothelial dysfunction[75].

Reduction in the synthesis or local availability of **NO** have been frequently considered the most important cause of endothelial dysfunction in various clinical conditions. NO release from the endothelium, is decreased in patients with established coronary atherosclerosis and hypertension[30,61,62]. A reduction in vascular availability of NO determines damage to the endothelium-dependent vasodilation, an increased tendency for platelet aggregation and adhesion of monocytes to the endothelium, thus influencing the proliferation of vascular smooth muscle cells, contributing to the onset and progression of hypertension.

NITRIC OXIDE AS THE MEDIATOR OF MANY THERAPEUTIC DRUGS :

The NO pathway contributes to the blood pressure lowering effects of the commonly used therapeutic agents. Emerging basic research on this pathway is elucidating potential new antihypertensive drug targets.

Some of the examples are:

1. HMG-CoA reductase inhibitors (statin drugs) have been shown to increase eNOS expression in the vessel wall[280-281] , which may account in part for the blood pressure lowering effects to a moderate extent .

2.Estrogen replacement therapy increases expression of both eNOS and nNOS in rats[282] . These effects could underlie some of the cardioprotective effects of estrogen and explain why postmenopausal estrogens do not cause hypertension.

3. Some of the antihypertensive action of ACE inhibitors is due to the decreased breakdown of bradykinin, resulting in receptor-mediated activation of eNOS and thus improved endothelial-mediated vasodilation[275].

4. In addition, excessive NO mediated vasodilation accounts for the marked and sometimes fatal hypotensive response to nitrates in patients receiving sildenafil, a phosphodiesterase inhibitor[283].

5. NO donors, such as nitroglycerin, cause vasodilation by increasing cGMP in the vessel wall and this increase is augmented when its breakdown is prevented by phosphodiesterase inhibition.[275, 283]

NITRIC OXIDE :

First studied in 1772 by Joseph Priestly, who called it "nitrous air," nitric oxide was first discovered as a colorless, toxic gas. It was through various researches it was found to be a signaling molecule and involved in various physiological functions .

- In the 1970s, Ferid Murad and his colleagues demonstrated that soluble guanylate cyclase (sGC) was stimulated by nitrogen-containing compounds, causing an increase in cGMP, which in turn brought about vascular relaxation [213,214].

- Murad first showed that the activation of sGC by nitrovasodilators could occur via the formation of NO. He was fascinated by the idea that a gas and free radical could regulate smooth muscle function and proposed that

hormones and other endogenous factors may also act through NO, which functions as a messenger, was proposed for the first time [215,216].

- In 1980, Furchgott and his colleagues published an article [217]underlining the obligatory role of endothelial cells in the acetylcholine-induced relaxation of arterial smooth muscle and recognized that vasodilation by bradykinin, histamine and adenosine diphosphate was due to the same relaxing substance, which they named endothelial derived relaxing factor (EDRF).

- It was Ignarro [211] who went on to conclude that EDRF from the artery and vein is either NO or a chemically related radical species in 1987 after Murad suggested [218]that, EDRF was an "endogenous nitrovasodilator". Subsequently, Salvador Moncada's group revealed that NO release accounts for the biological activity of EDRF[212].

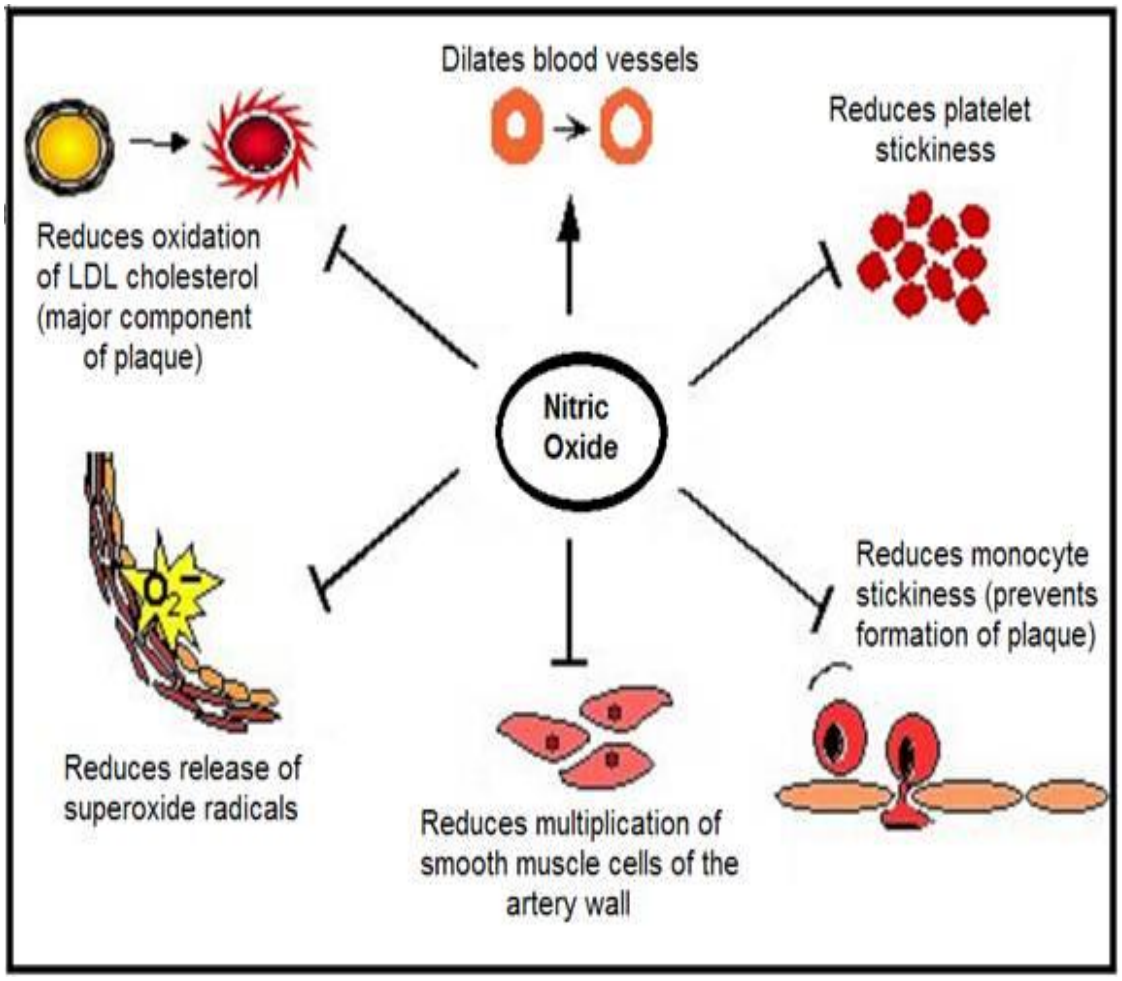
- By the early 1980's, scientists had conclusively proven that, NO occurred naturally within the human body. By 1987, its role in regulation blood pressure and relieving heart conditions was well-established. Two years later, research revealed that NO is used by macrophages to kill tumor cells and bacteria.

- In 1992**, NO** was voted "**Molecule of the Year**" by Science magazine.

- On October 12, 1998, the Nobel Assembly awarded the Nobel prize in Medicine or Physiology to scientists Robert Furchgott, Louis Ignarro, and Ferid Murad for their discoveries concerning NO as a signaling molecule in

Figure :6

ACTIONS OF NITRIC OXIDE ON VASULATURE



the cardiovascular system, opening up a new way of treatment for millions of patients.

- In 1999, it was reported that, the most highly cited U.K. biomedical scientist of that decade was S. Moncada, because of the work of his group on NO .

- Now, in 2010, more than 70,000 scientific papers have been published on NO and its seemingly endless role in health and physiology.

NITRIC OXIDE PATHWAY :

NO is produced by the enzyme nitric oxide synthase (NOS) [275] which catalyzes a five electron oxidation of a guanidino nitrogen of L-arginine (L-Arg). Oxidation of L-Arg to L-citrulline occurs via two successive mono-oxygenation reactions producing N^Ghydroxy L-arginine as an intermediate. Two moles of O_2 and 1.5. moles of NADPH are consumed per mole of NO formed [213] . NO has various physiological actions as shown in Figure :6

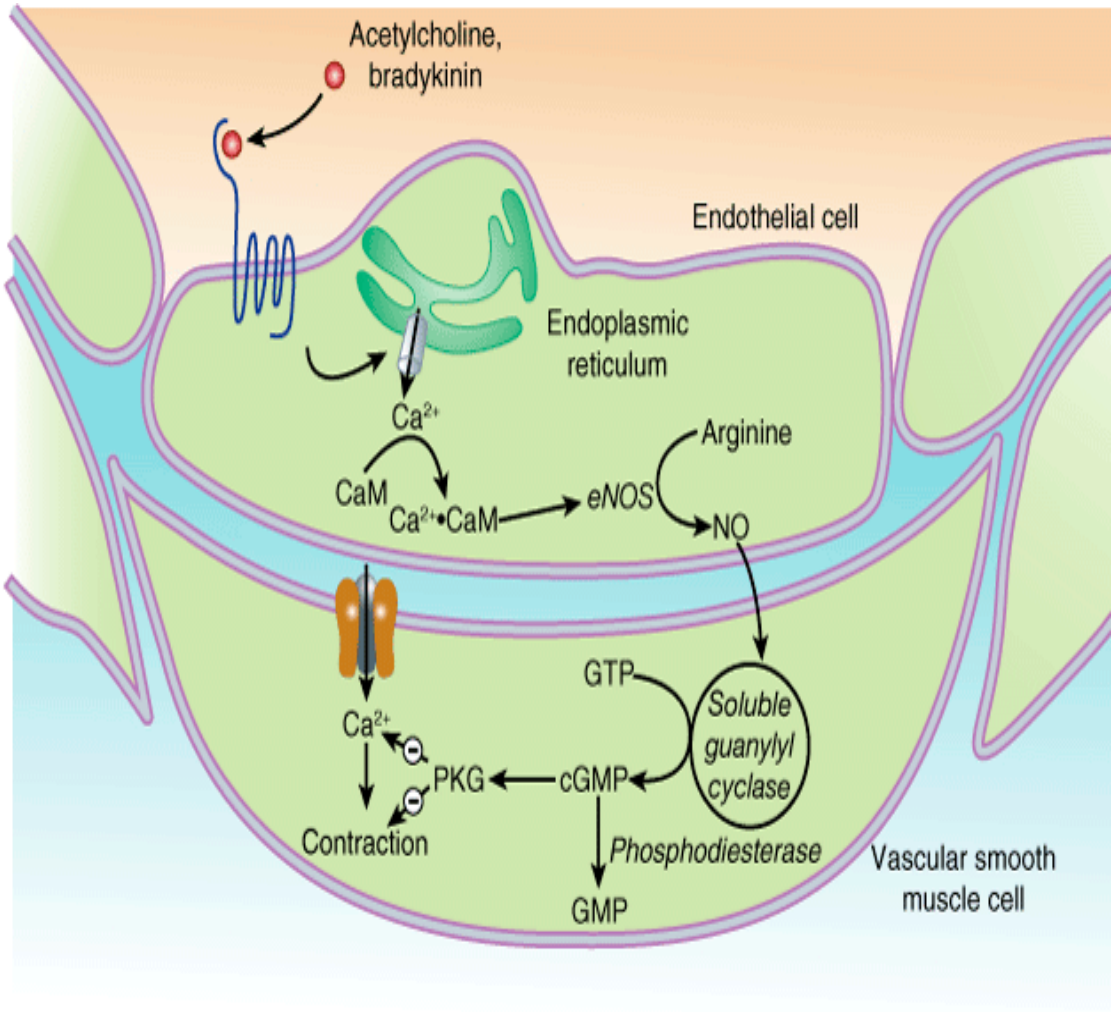
NO BASED CELL SIGNALING :

I. cGMP dependent signaling (Figure : 7)

NO diffuses into the nearby target cells to interact with specific molecular targets. NO regulates protein activity by reversibly binding to the available acceptor functionalities, including heme iron and thiols [297]The interaction between NO and the enzyme guanylyl cyclase, which mediates target cell responses such as vascular smooth muscle relaxation and platelet inhibition, has been well characterized [298,299]. After entering the target cell, NO binds to the heme moiety of guanylyl cyclase and

Figure : 7

cGMP DEPENDENT NITRIC OXIDE PATHWAY :



activates the enzyme by inducing a conformational change that displaces iron out of the plane of the porphoryrin ring [300] Guanylyl cyclase then catalyzes the production of cyclic GMP from GTP to elevate cyclic GMP, which triggers a cascade of intracellular events that brings about a reduction in calcium-dependent vascular smooth muscle tone, by inactivating myosin light chain kinase (MLCK) [301,302]. MLCK normally phosphorylates the regulatory set of myosin light chain. This event activates cross-bridge cycling and initiates contraction [303]. cGMP modulates MLCK activity by activating a cGMP-dependent protein kinase that phosphorylates MLCK . Phosphorylation of MLCK diminishes its affinity for calmodulin and, as a consequence, decreases the phosphorylation of myosin light chain, which in turn stabilizes the inactive form of myosin. In this manner, cGMP may induce vasorelaxation by indirectly decreasing myosin light chain-dependent myosin activation.

GUANYLYL CYCLASE :

Soluble GC(sGC) is a heme-containing, heterodimeric NO receptor. sGC consists of two subunits, α and β, which make up the active enzyme. sGC isoforms, products of four genes, have been identified so far as α_1, α_2, β_1 and β_2. Only α_1/β_1 and α_2/β_1 heterodimers are activated by NO [220]. The α_1/β_1 sGC is the most abundant isoform [221]. Vascular smooth muscle and endothelial cells express predominantly α_1 and β_1 subunits [222]. The functional importance of α_1/β_1 sGC was demonstrated by the significantly decreased relaxing effects of major vasodilators such as acetylcholine, NO, YC-1 and BAY 41-2272 in the α_1 sGC knockout mice[223] . Through the production of cGMP, sGC can exert many physiological effects such as mediating

vascular smooth muscle tone and motility, phototransduction, and maintaining fluid and electrolyte homeostasis [224-226]. The sGC activity increases more than 200 fold in response to NO[227,228]. This signal is quickly removed by the action of phosphodiesterase 5A enzyme.

PHOSPHODIESTERASE INHIBITORS :

Phosphodiesterases (PDEs) are intracellular enzymes that specifically catalyze the hydrolysis of the second messengers cAMP and cGMP to the inactive metabolites AMP and GMP. By counterbalancing cGMP production by guanylyl cyclases, PDE5 is able to decrease cGMP levels very effectively. Thus PDE5 inhibition increases intracellular cGMP levels and initiates a cGMP-driven cascade of reactions [224]. Inhibitors of PDE5A such as sildenafil are widely used to treat erectile dysfunction, but growing evidence supports important role of the enzyme in both the vasculature and heart. These agents may also be beneficial in other disorders, such as pulmonary hypertension, Raynaud's syndrome, etc.

SPLICE FORMS AS NOVEL GENETIC REGULATORS OF sGC :

Recently, the vital importance of sGC for mammalian physiology was directly confirmed by generation of sGC knockout mice [223,229,230]. The absence of sGC protein resulted in a significant increase in blood pressure, complete loss of NO-dependant aortic relaxation and platelet aggregation in knockout animals, which died prematurely at the age of 4 weeks due to severe gastrointestinal disorders [229]. sGC function is affected not only by NO, but also by regulation of the expression of sGC subunits at transcriptional and post-transcriptional levels. The steady state

mRNA levels of α_1 and β_1 subunits decrease with hypertension, ageing and vary during embryonic development [231]. The expression of sGC subunits is regulated by estrogen [232], cAMP-elevating compounds [233,234], cytokines [235] and NO donors [236]. Gene therapy with α_1 β_1 subunits may provide future therapeutic utility in hypertension.

ALLOSTERIC EFFECTORS OF sGC :

There are many allosteric regulators of sGC which provide NO independent activation. Current therapies that involve the use of organic nitrates and other NO donors have limitations, including non-specific interactions of NO with various biomolecules, lack of response and the development of tolerance following prolonged administration. Compounds that activate sGC in an NO-independent manner might therefore provide considerable therapeutic advantages [238]. NO-independent but heme-dependent stimulators of sGC areYC-1, BAY 41-2272, BAY 41-8543, A-350619 and CFM-1571. NO as well as heme-independent sGC activators are BAY 58-2667 and HMR-1766.

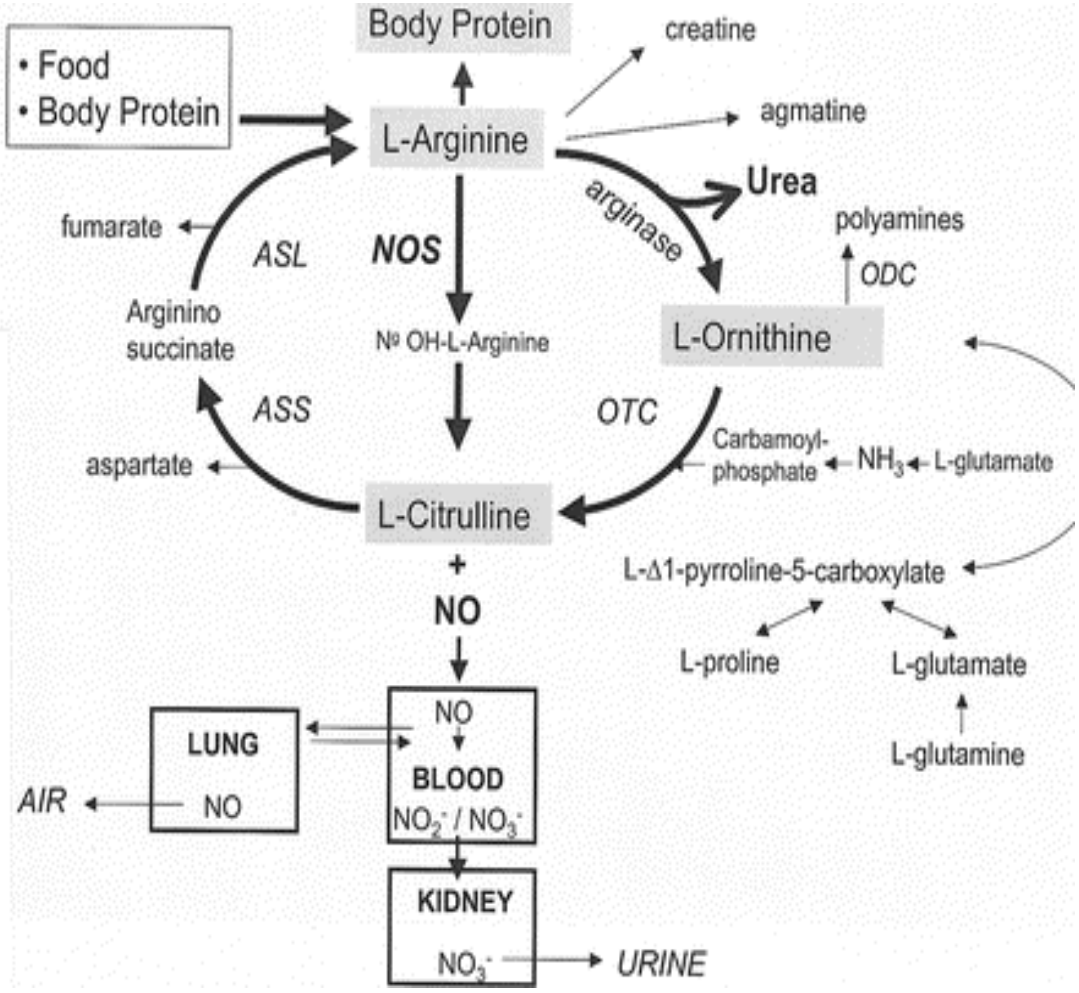
II. cGMP INDEPENDENT SIGNALING :

A. Nitrite and nitrate (Figure : 8):

Nitrite is an oxidative breakdown product of NO that has been shown to serve as an acute marker of NO formation [243]. Nitrite has recently moved to the forefront of NO biology [244] as, it represents a major storage form of NO in blood and tissues [245] Much of the recent focus on nitrite physiology is due to its ability to be

Figure : 8

NITRIC OXIDE END PRODUCTS – NITRATE AND NITRITE



reduced to NO [239-242], during ischemic or hypoxic events [245]. They get converted to NO and exerts its physiological events . Evidences for this fact include :

- enriching dietary intake of nitrite and nitrate translates into significantly less injury from heart attack [239].
- nitrite therapy given intravenously prior to reperfusion protects against myocardial injury [251,252] and cerebral vasospasm [253].
- inhalation of nitrite selectively dilates the pulmonary circulation under hypoxic conditions *in vivo* in sheep [254].
- topical application of nitrite improves skin infections and ulcerations [255].
- oral nitrite has also been shown to reverse L-Nitro Arginine Methyl Ester (L-NAME) induced hypertension and serve as an alternate source of NO *in vivo* [256].
- plasma nitrite levels progressively decrease with increasing cardiovascular risk.[257]

In addition to the oxidation of NO, nitrite is also derived from reduction of salivary nitrate by commensal bacteria in the mouth and gastrointestinal tract [246,247]as well as from nitrite rich [240] diet. Since a substantial portion of nitrite in blood and tissue are derived from dietary sources [240], modulation of nitrite and/or nitrate intake may provide a first line of defense for conditions associated with NO insufficiency [245]. The primary dietary sources of nitrates and nitrites include plants, vegetables and a few fruits, nuts, processed and cured meat, fish and poultry and drinking water, to which nitrites have been added. Plant foods are the primary

sources of nitrate, while processed and cured meats are the primary sources of nitrites [258]

Since the **NO•** radical is rapidly metabolized into the stable end-products nitrite and nitrate, determination of nitrite and nitrate does not demonstrate ongoing NO• production. Considering that the half-life of nitrate in plasma is ~5 h [195], increased concentrations of nitrate in plasma after overnight fasting at least indicate recent NO• production. The most commonly used nitrite assay is based on the Griess diazotization reaction, which is specific for nitrite and does **no**t detect nitrate. Therefore, nitrate in samples must first be reduced to nitrite; subsequent nitrite determination thus yields the total nitrite + nitrate concentration of the sample (NOx)

Various studies on plasma NOx assay[203]:

(a) recovery of nitrite and nitrate from plasma is near-quantitative (87%) and reproducible.

(b) nitrite and nitrate are stable in (frozen) plasma for at least 1 year.

(c) nitrite in whole blood is very rapidly (> 95% in 1 h) oxidized to nitrate**,** and therefore plasma nitrite determination alone is meaningless.

(e) plasma nitrite and nitrate concentrations were not correlated (nitrite a**s** % of total nitrite + nitrate varied from 3.9% to 88% in plasma samples).

(f) plasma samples should be de-proteinized, and background controls should be included in the assay, to avoid measuring the falsely high nitrite and nitrate concentrations in plasma.

b. S-NITROSOTHIOLS :

S-nitrosothiols are thio-esters of nitrite, with the general structure R-S-N=O; naturally occurring examples include S-nitrosocysteine, S-nitrosoglutathione and S-nitrosoalbumin, in which R is an amino acid, polypeptide and protein respectively. Reactive protein thiols are regarded as major intracellular target of NO. Nitrite is in steady state of equilibrium with S-nitrosothiols .

S-nitrosation has since been implicated in the control of a wide array of protein functions and cell activities like, regulation of apoptosis, G-protein-coupled receptor based signaling, vascular tone and inflammatory responses [262-265]. Among the growing list of proteins whose activities are regulated by s-nitrosation some are, ion channel proteins, kinases, proteolytic enzymes, transcription factors and proteins involved in energy transduction [259]. Dysregulation of protein S-nitrosation is associated with a growing list of pathophysiological conditions [259]and altered blood levels of RSNO have been associated with impaired clinical outcome in patients with cardiovascular disease[260,261].

S-nitrosoglutathione reductase (GSNOR), a member of alcohol dehydrogenase family, has been shown to be the primary pathway through which cells denitrosate intracellular proteins [266]. GSNOR has become an important target for developing agents that modulate NO bioactivity inside the cells.

3. NITROTYROSINE :

NO reacts with superoxide anion to form peroxynitrite, that can further form peroxynitrous acid, a very unstable and reactive oxidizing species. Involvement of ONOO is the most widely studied mechanism of protein nitration [267] . The formation of nitrotyrosine has been detected in various pathological conditions including atherosclerosis, myocardial infarction, hypertension, myocarditis, heart failure, shock, diabetic complication and neurodegenerative and inflammatory disorders [268].

Substantial evidence has emerged which revealed a very close association between the formation of nitro-tyrosine and the presence of activated granulocytes containing peroxidases, such as MPO [269-272]. MPO-generated reactive species participate in the induction of foam cell formation, endothelial dysfunction, and development of vulnerable plaque. Carr and Frei have revealed that, physiological concentrations of nitrite inhibit MPO mediated modification of LDL[273]providing a means to interrupt the process. These data also demonstrate the first line of evidence of nitrite acting in an "antioxidant" capacity in atherosclerosis.. This may represent a novel mechanism by which metabolites of NO may exert an anti-atherogenic effect .

REDUCED NITRIC OXIDE IS NOT DUE TO SUBSTRATE[296] :

In normal humans, availability of substrate for production of No, is a rate-limiting step for endothelium-dependent vascular relaxation. In contrast, increased availability of NO precursor does not modify endothelium-mediated vasodilation in hypertensives. These findings provide evidence of a defect in the endothelium-

derived NO system in hypertension and indicate that, this abnormality is not related to decreased availability of substrate for NO production.

SUPEROXIDE PRODUCTION AS A CAUSE OF NITRIC OXIDE-DEFICIENT HYPERTENSION : (Figure : 9)

One of the principal mechanisms of endothelial cell dysfunction in hypertension, is the production of superoxide anion and other reactive oxygen species that quench NO, thereby reducing its bioavailability[63]. There are three main enzymatic sources of vascular superoxide:

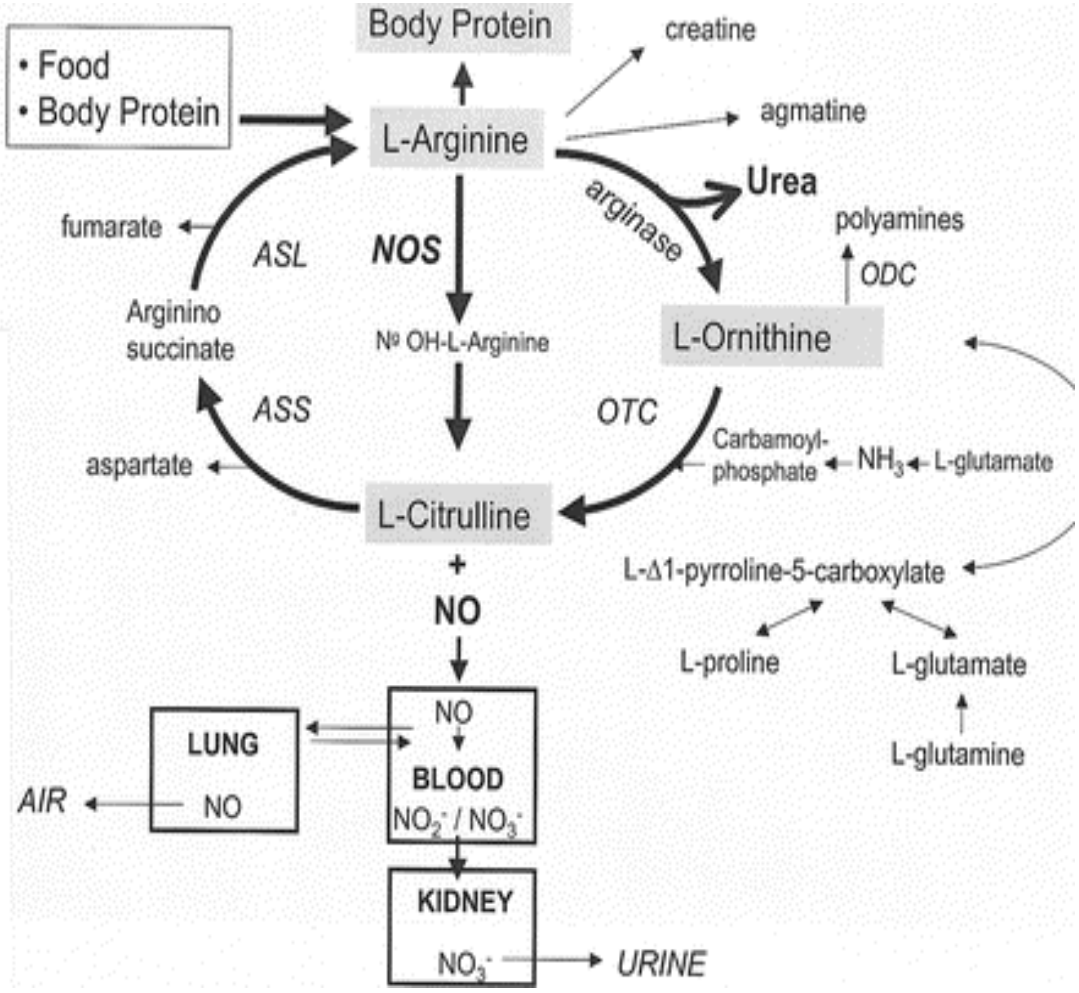
(1) Reduced form of NADPH oxidases, The activity of NAD(P)H oxidases is increased by physical stress (eg, increased blood pressure), cytokines and angiotensin II[220]**.** There is increasing experimental evidence that, generation of superoxide anion by NAD(P)H oxidases is a major mechanism by which, minimal elevations in plasma renin and angiotensin II levels lead to sustained elevations in blood pressure[288] . The reduced generation of superoxide and restoration of endothelial-dependent vasodilation appears to be an important mechanism mediating the anti-hypertensive and cardioprotective effects of ACE inhibitors and angiotensin-receptor blockers[289-290].

(2) NOS produces superoxide, only when an important cofactor (tetrahydrobiopterin) is deficient (a process known as NOS uncoupling).

(3) Xanthine oxidase, that produces uric acid[76]. Generation of reactive oxygen species by xanthine oxidase accounts for the association of elevated serum uric acid levels with endothelial dysfunction and essential hypertension[77]. However,

Figure : 8

NITRIC OXIDE END PRODUCTS – NITRATE AND NITRITE



uric acid is an antioxidant too. Thus, the xanthine oxidase inhibitor allopurinol, does not consistently lower blood pressure in patients with hypertension, as the drug simultaneously reduces both these opposing effects on vascular function[78].

Experimental studies suggest that the antioxidant agents may re-establish endothelial function[64,65]. Vitamin C, a potent antioxidant in vivo and in vitro [66] inhibits superoxide-mediated lipid peroxidation[67] and improves endothelial function in smokers[70] as well as in patients with coronary artery disease[68] and diabetes mellitus[69]. Superoxide dismutases are enzymes produced in blood vessels that normally inactivate superoxide. In rats, hypertension produced with infusion of angiotensin-II, was reversed when superoxide dismutase was delivered in membrane-permeable liposomes[289]. Increased antioxidant production with improved endothelial-dependent vasodilation is likely to be a potential mechanism, underlying the mild antihypertensive effect of regular aerobic exercise[293].

DECREASED NOS EXPRESSION AS A CAUSE OF HYPERTENSION [294] :

The eNOS knockout mouse develops mild-to-moderate hypertension, with basal blood pressure levels typically 20 mm Hg higher than in control littermates[276]. When normotensive mice were engineered to express the constitutively active eNOS in excess, their mean blood pressure levels were 18 mm Hg lower than normal[278]. In spontaneously hypertensive rats, a single intravenous injection of human complementary DNA encoding eNOS almost completely normalized blood pressure for 5 to 6 weeks[279].

ENDOGENOUS NOS INHIBITORS AS A CAUSE OF HYPERTENSION :

An increase in endogenous inhibitors of nitric oxide synthesis, may be involved in the genesis of endothelial dysfunction. In normotensive animals and humans, administration of methylated arginines, which are competitive NOS inhibitors[276-277], caused marked and dose-dependent elevations in blood pressure level.

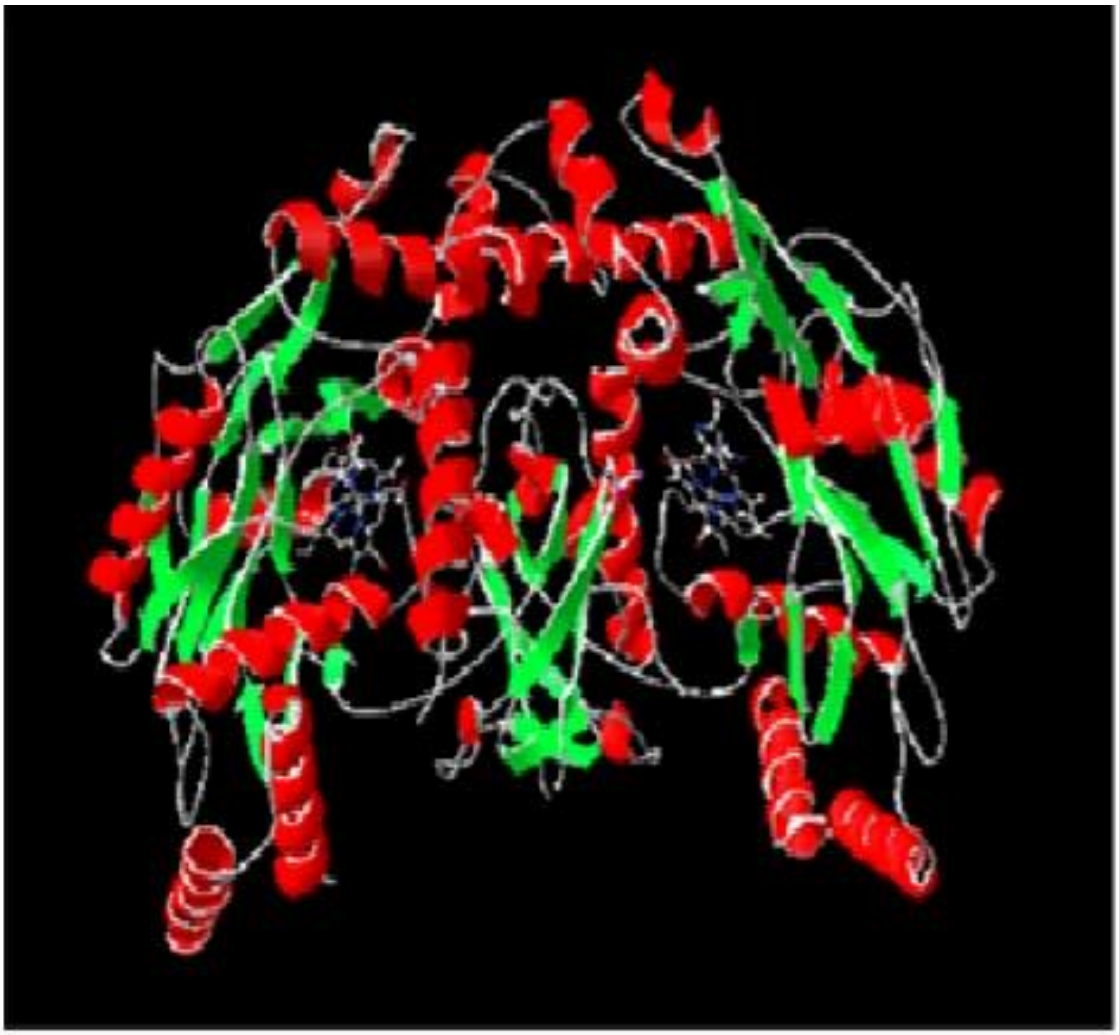
Asymmetric dimethyl arginine (ADMA) and symmetric dimethyl arginine are endogenous substances first isolated from human plasma and urine. When infused into the human brachial artery, ADMA causes vasoconstriction whereas symmetric dimethyl arginine is inert[284]. Because ADMA is normally cleared by the kidney, the plasma concentration increases 3- to 10-fold in humans and animals with renal failure[285]. It competes with L-arginine to prevent the synthesis of nitric oxide [71]. Thus, accumulation of this endogenous NOS inhibitor has been hypothesized to contribute to hypertension in patients with chronic renal failure[287](but not yet proven). Studies have shown reduced urinary excretion of NO metabolites and increased plasma levels of ADMA in men with essential hypertension[285].

NITRIC OXIDE SYNTHASES (NOSS, EC 1.14.13.39) :

The NOSs were first identified and described in 1989. The three major isoforms were cloned and purified between 1991 and 1994. The first X-ray crystal structures of NOS domains have been presented and published in 1998 and 1999 (Figure : 10) .

Figure : 10

X-RAY CRYSTALLOGRAPHIC STRUCTURE OF ENDOTHELIAL NITRIC OXIDE SYNTHASE



Three quite distinct isoforms of NOS have been identified as products of different genes, with different localization, regulation, catalytic properties, inhibitor sensitivity and with 51–57% homology between the human isoforms. These isoforms will be referred to, by the most common nomenclature , based on the order in which they were first purified and cloned.

- nNOS (also known as Type I, NOS-I and NOS-**1**) being the isoform first found (and predominating) in neuronal tissue
- iNOS (also known as Type II, NOS-II and NOS-2) being the isoform which is inducible in a wide range of cells and tissues
- eNOS (also known as Type III, NOS-III and NOS-3) being the isoform first found in vascular endothelial cells

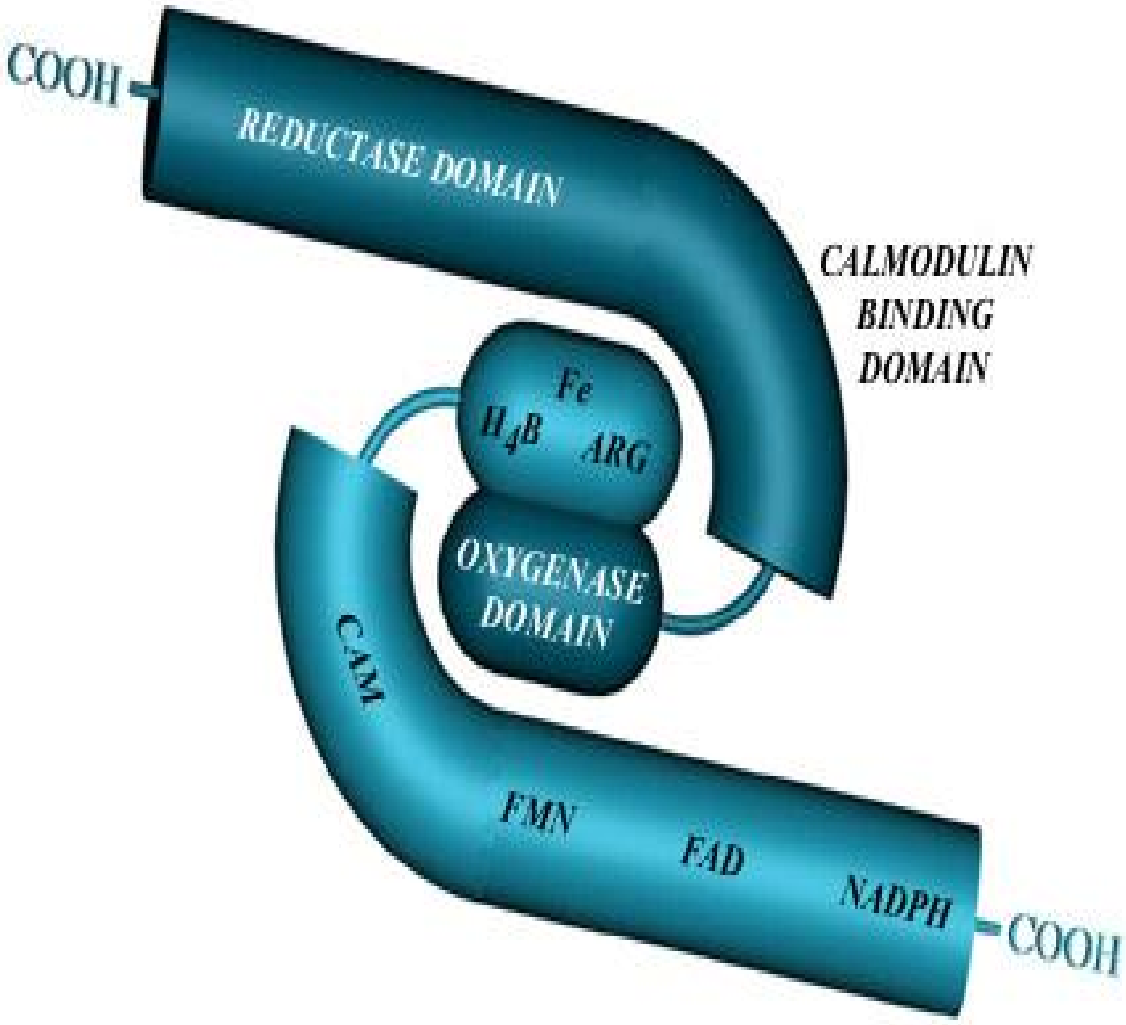
In the past, these isoforms have also been differentiated on the basis of their constitutive (eNOS and nNOS) versus inducible (iNOS) expression and their calcium-dependence (eNOS and nNOS) or independence (iNOS).

STRUCTURE : (Figure : 11)

NOS exhibit a bi-domain structure, in which an N-terminal oxygenase domain containing binding sites for heme, BH_4 and L-arginine, is linked by a calmodulin-recognition site to a C-terminal reductase domain, containing binding sites for FAD, FMN and NADPH [83-85]. The known NOS enzymes are usually referred to as 'dimeric' in their active form, ignoring the required calmodulins (CaMs) which, strictly speaking, mean they are tetramers (of two NOS monomers

Figure : 11

VARIOUS DOMAINS OF ENDOTHELIAL NITRIC OXIDE SYNTHASE



associated with two CaMs). They contain relatively tightly bound cofactors BH4, FAD, FMN and heme.

NOS dimerization:

The association of the NOS into active dimers, involves a large interface in the oxygenase domain, involving two sections of the primary structure of NOS [86,87,89] . This interface includes the binding site for BH4 and helps to structure the active-site pocket containing the heme and the L-arginine binding site; it has two cysteine residues per monomer, which either forms a disulphide bridge between the monomers or ligates a zinc ion between the monomers [88,89]. Furthermore, there is an 'N-terminal hook' domain, which swaps between the two monomers, to stabilize the dimer [89]. BH4 as well as heme and L-arginine promote and stabilize the active dimeric form of all the three isoforms. The presence of heme appears to be mandatory, with BH4 and L-arginine promoting dimer formation and stabilization. The flow of electrons from reductase domain of one monomer, to the oxygenase domain of the other monomer forms NO, as shown in Figure: 12.

Endothelial cell constitutive nitric oxide synthase can be activated by stimuli that include thrombin, adenosine diphosphate, bradykinin, substance P, muscarinic agonists, catecholamines, and shear stress [121].

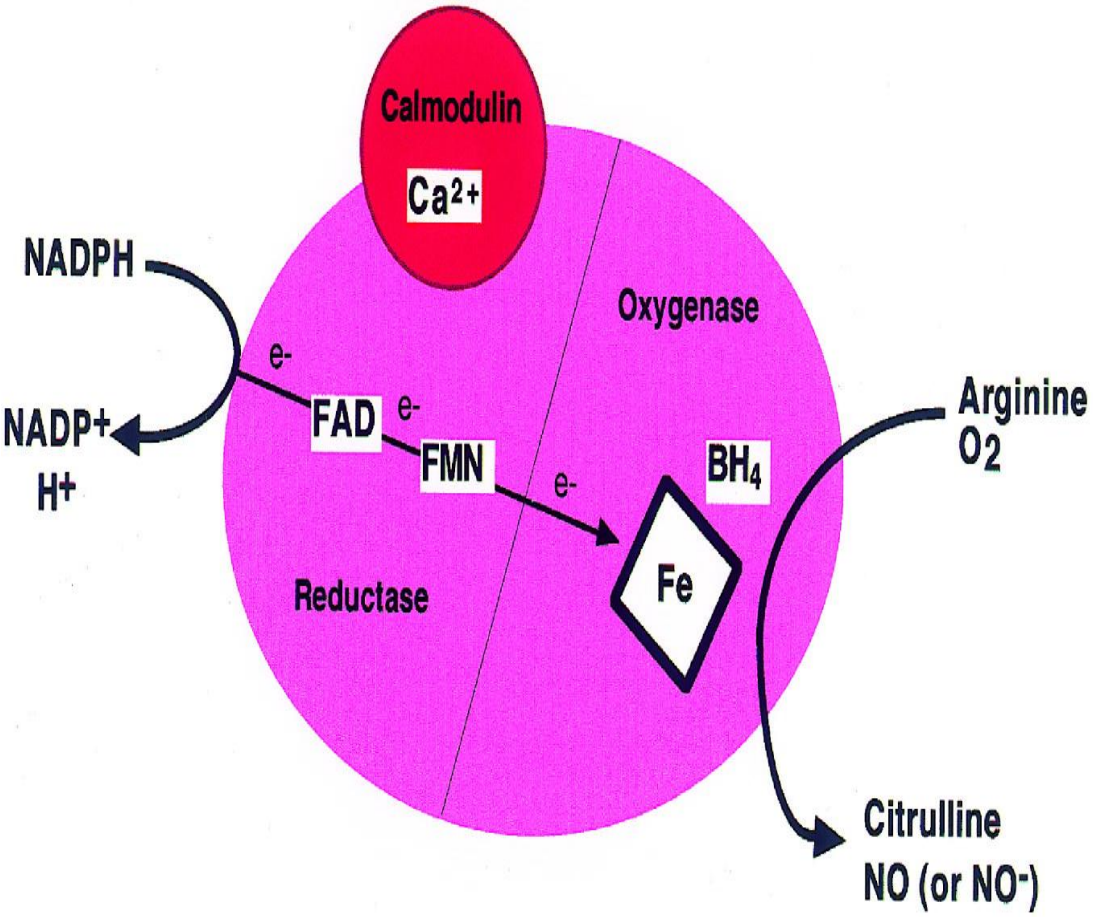
REGULATION OF NOS :

CALMODULIN:

Calmodulin was the first protein shown to interact with NOS[90] and is necessary for the enzymatic activity of the three isoforms. CaM binding increases

Figure 12 :

ELECTRON FLOW IN eNOS



the rate of electron transfer from NADPH to the reductase domain flavins [91,92]and from the reductase domain to the heme centre [93].

PHOSPHORYLATION:

Phosphorylation of the nNOS and eNOS isoforms has an effect on NOS activity. Fluid shear stress elicits phosphorylation of eNOS and an increase in its activity [94,95]. Studies showed that Ser^{1179} of eNOS is phosphorylated by protein kinase [96,97], which results in an increase in electron flux through the reductase domain and an increase in NO production [98].

HEAT-SHOCK PROTEIN 90 (hsp90):

The molecular chaperone hsp90 has been identified as a regulator of eNOS activity, possibly as an allosteric modulator [103]. Activation by vascular endothelial growth factor, histamine or fluid shear stress in human endothelial cells increases the interaction between eNOS and hsp90 and increases eNOS activity by approximately three-folds. . Kallikrein appears to inhibit iNOS by preventing the formation of iNOS dimers [104] and may play a neuroprotective role during inflammation**.**

REGULATION OF eNOS LOCALISATION: (Figure : 13 a & b)

MYRISTOYLATION and PALMITOYLATION :

Of the three NOS isoforms, only eNOS is acylated by both myristate and palmitate [105]. eNOS is co-translationally and irreversibly myristoylated at an N-terminal glycine residue while palmitoylation occurs post-translationally and reversibly at cysteine residues (Cys^{15} and Cys^{26}). Dual acylation of eNOS is required

Figure : 13a

REGULATION OF eNOS LOCALISATION – MYRISTOYLATION & PALMYTOYLATION

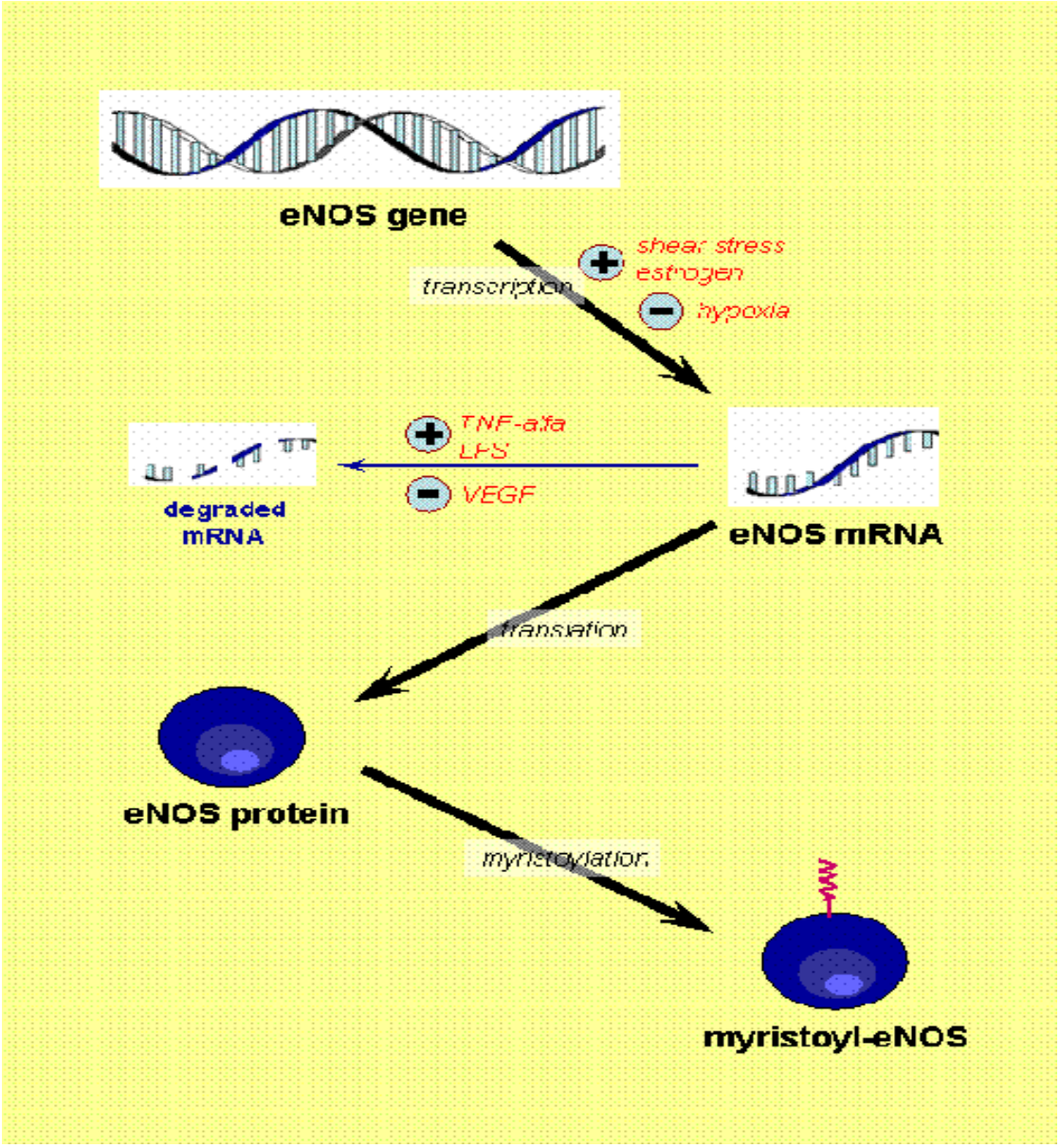
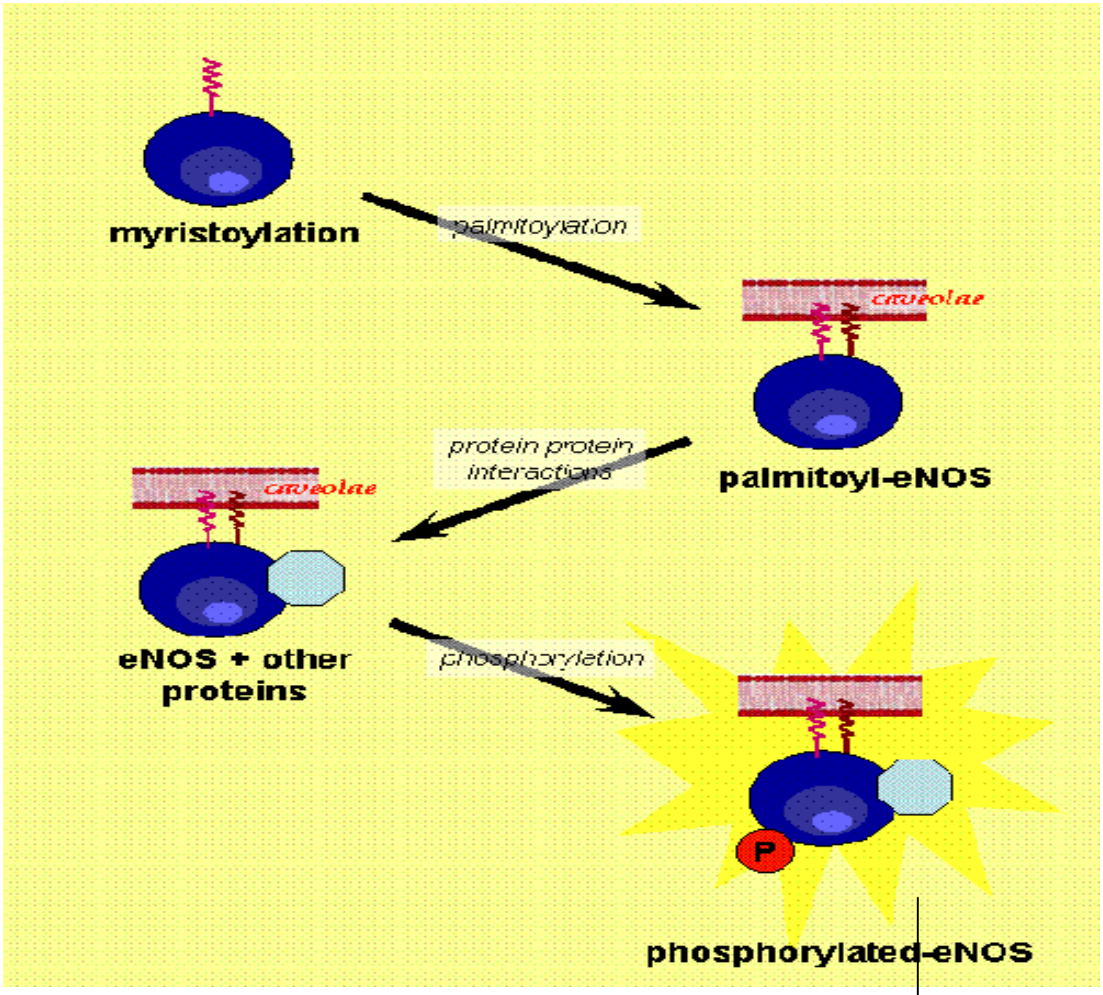


Figure : 13b

REGULATION OF eNOS LOCALISATION – MYRISTOYLATION & PALMYTOYLATION



for efficient localization to the plasmalemmal caveolae of endothelial cells [108]. Palmitoylation[109] is dynamically regulated by bradykinin -induced changes in intracellular Ca^{2+}.

CAVEOLIN :

eNOS is localized to the caveolae[108,110] , which are microdomains of the plasmalemmal membrane that are implicated in a variety of cellular functions including signal transduction events. Vascular eNOS binds to caveolin-1, while in cardiac myocytes , eNOS is associated with caveolin-3 [111]. Caveolin-1 and peptides from the 'caveolin-1 scaffold region' directly inhibit eNOS activity and this interaction is regulated by Ca^{2+}/CaM [112,113]. Caveolin-3 binds to nNOS in the skeletal muscle, inhibiting NO synthesis and this inhibition is reversed by Ca^{2+}/CaM [114].

MITOCHONDRIAL NOS :

There have been some suggestions in recent years that, there might be a NOS in mitochondria. This was originally based on cytochemical and immuno-cytochemical evidence [116-118] , showing association of either NADPH diaphorase or NOS antibody-binding to mitochondrial membranes. This NOS seems likely to consist of a membrane-associated iNOS[115]. Although iNOS does not have fatty acylation sites like those of eNOS, membrane association via binding to membrane proteins such as the caveolins provide a plausible alternative mechanism for this.

PHYSIOLOGICAL ROLE OF NOS:

- Under normal physiological conditions, iNOS is not detectable in macrophages and other tissues, and thus appears to play little if any role in the normally functioning cardiovascular system. However, induction of iNOS expression by cytokines or inflammatory mediators is thought to play a pivotal role in causing hypotension during septic shock.
- In contrast, both eNOS and nNOS are constitutively expressed and are thought to contribute to the normal regulation of vasomotor tone and blood pressure[119]. eNOS derived NO was considered to be the most important regulator of vasomotor tone[120].

eNOS AND VASOMOTOR TONE :

In the vascular endothelium, agonists such as acetylcholine and bradykinin stimulate inositol 1,4,5-triphosphate (IP_3) production, the second messenger system. IP_3 binds to receptors on the endoplasmic reticulum and causes Ca^{2+} release from intracellular stores [122]. This transient elevation of intracellular Ca^{2+} promotes calcium binding to calmodulin, forming a complex that is a crucial cofactor for constitutive NOS activity [123]. eNOS produces modest amounts of NO until the calcium concentration decreases. This rapid and transient production of NO by eNOS allows NO to function in maintaining basal vascular tone [122].

eNOS GENE :

Given the pivotal role of eNOS in vascular homeostasis, the eNOS gene (NOS3) has emerged as a logical candidate gene in the investigation of hypertension genetics [126].

Animal models of altered eNOS gene expression have emphasized the crucial role of this enzyme in a diverse array of vascular biology and pathology. Among others, $eNOS^{-/-}$ (knock out) mice have been associated with systemic and pulmonary hypertension[124-126], altered vascular remodeling[127,128] , impaired angiogenesis[129,130] and perturbations in homeostasis. A decrease in eNOS protein expression was also observed by immunohistochemistry[131] . This down-regulation of eNOS mRNA, undoubtedly, contributes to the reduced endothelial NO production and defective endothelium-dependent vasorelaxation[133] observed in diseased atherosclerotic vessel. It is now appreciated that decreased endothelial eNOS mRNA and protein can be observed in diseased human blood vessels .

NOS3 gene is located in the chromosome 7 , corresponding to the 7q35-q36 region, contains 26 exons with an entire length of 21kb. (Figure :14)

REGULATION OF eNOS GENE EXPRESSION :

Numerous exogenous stimuli and conditions that are relevant to the pathology of vascular endothelium have been shown to alter eNOS expression through the modulation of steady-state eNOS mRNA [134]. This regulation occurs at both the transcriptional and posttranscriptional levels.

TRANSCRIPTIONAL REGULATION:

eNOS CORE PROMOTER:

The eNOS gene encodes a mRNA of 4052 nucleotides and is present as a single copy in the haploid human genome. Sequence inspection of 5'-flanking regions revealed multiple potential *cis*-regulatory DNA sequences in the setting of a

Figure : 14

CHROMOSOMAL LOCALISATION OF eNOS

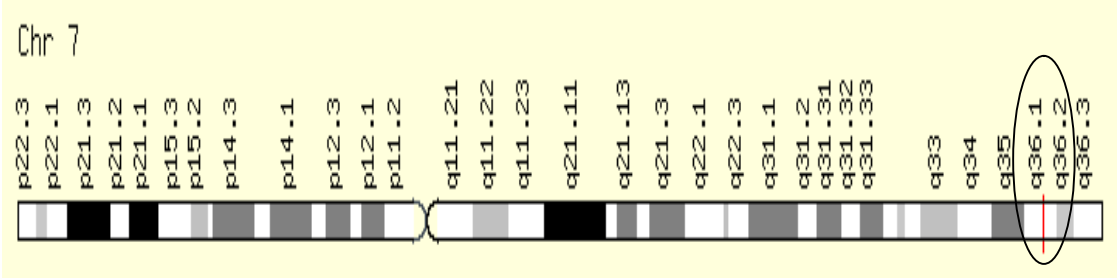
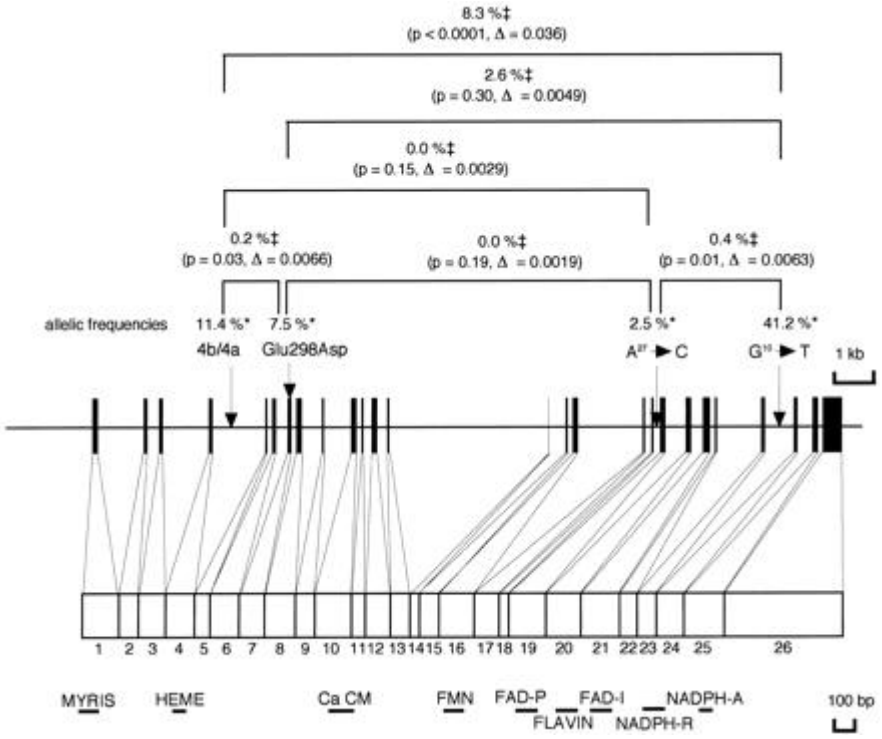


Figure : 15

VARIOUS POLYMORPHISMS OF eNOS



"TATA-less" promoter: Sp1, GATA, AP-1, NF-1, shear-stress response elements, and sterol-regulatory elements[135]. There are two tightly clustered *cis*-positive regulatory elements in the proximal core promoter of the human eNOS gene. **Positive regulatory domain I** (PRD I; -104 to -95 relative to transcription initiation) was mapped to a 10-bp *cis*-region corresponding to a high-affinity Sp1 transcription factor recognition site. **PRD II** (-144 to -115) encompassed a 30-bp region of the core promoter and formed nucleoprotein complexes containing the transcription factors Elf-1, YY1, Sp1, and MAZ[136]. Expression studies in *Drosophila* SL2 cells revealed that functional interactions between these *trans*-acting factors are critical for eNOS promoter activation. Mutating *cis*-elements in PRD I or PRD II disrupted co-operative activation of the human promoter[136] .

CELL SPECIFIC EXPRESION :

eNOS gene expression is relatively restricted to the vascular endothelium[134]. The existence of a distal enhancer element has the ability to direct the appropriate cell-specific expression pattern of the eNOS gene. The human eNOS proximal promoter was differentially methylated in expressing and non-expressing cell types[137]. In non-expressing cells, the core promoter CpG di-nucleotides were densely methylated, whereas in eNOS-expressing endothelial cells they were non-methylated. Differential promoter methylation was further implicated in the determination of cell-specific eNOS expression, by the successful induction of endogenous eNOS mRNA expression in non-expressing cell types. This may occur in response to de-methylation by 5-azacytidine, a DNA methyltransferase inhibitor. It has been suggested that promoter methylation down-regulates transcription by

recruitment of methyl-CpG binding proteins and histone de-acetylase activity, resulting in a closed and transcriptionally repressive chromatin structure[138].

SHEAR STRESS :

The effect of changes in blood flow on eNOS expression has been an important focus of investigation[139]. Animal studies emphasized the role of the endothelium in exercise and flow induced vasodilation, which showed an up-regulation of eNOS expression[140,141] . Indeed, $eNOS^{-/-}$ mice were found to be incapable of vascular remodeling, in response to shear stress[127]. At the molecular level, shear stress was found to induce eNOS mRNA expression via a transcriptional pathway[142].

TRANSFORMING GROWTH FACTOR (TGF-β1) :

TGF-β1 is increasingly recognized as an important mediator in vascular immune injury, vessel remodeling, vasculogenesis and angiogenesis[143,144]. TGF-β1 has been shown to increase bovine aortic endothelial cell (BAEC) and human umbilical vein endothelial cell (HUVEC) steady-state eNOS mRNA expression in a concentration-dependent manner[145,146] . The TGF- β response element was mapped to a NF-1 *cis*-DNA binding site, a region spanning -1000/-720 of the human promoter. TGFβ1 transactivates the eNOS promoter via recruitment of ribonucleoprotein (RNP) complexes containing SMAD2 and NF-1 at distinct sites.

CYCLOSPORINE A :

Immunosuppressive therapy with cyclosporine A has been commonly associated with hypertension, with its effects attributed to paracrine vasoconstrictors.

It was also found to augment NO production paradoxically in vitro and in vivo. Cyclosporin A treatment of endothelial cells in culture was shown to enhance eNOS expression through transcriptional activation[147,148] .

POST TRANSCRIPTIONAL REGULATION :

TUMOR NECROSING FACTOR (TNF-α) :

TNF-α has been shown to reduce endothelium-dependent vaso-relaxation in vivo and ex vivo[151,152]. Some of the studies of eNOS gene regulation, identified modulation of mRNA stability as a regulatory target of pro-inflammatory cytokines[151,154] . Indeed, TNF-α alone reduced the half-life of eNOS mRNA from 48 hours to 3 hours[149] .

DUAL REGULATION:

TRANSCRIPTIONAL AND POSTTRANSCRIPTIONAL EFFECTS:

OXIDIZED LOW-DENSITY LIPOPROTEINS :

Liao et al first reported that, oxidized low-density lipoproteins (ox-LDL) caused a time and concentration-dependent decrease in steady-state eNOS mRNA and enzyme activity in human endothelial cells[155]. This down-regulation was found to occur principally at the post-transcriptional level, resulting in a reduction in eNOS mRNA half-life from 36 to 10 hours. Low concentrations of ox-LDL (<10 µg protein/mL) may be associated with a paradoxical increase in eNOS mRNA and protein expression[156] . Very high levels of native LDL were found to decrease eNOS mRNA expression[157]. Lysophosphatidyl choline, a major component of ox-LDL, can

increase eNOS mRNA in BAEC[156]. Perhaps this provides an explanation for the biphasic effect of varying doses of ox-LDL.

3-HYDROXY-3-METHYLGLUTARYL-COENZYME A (HMG-CoA) REDUCTASE INHIBITORS (STATINS):

The mechanisms, by which statins confer lipid-independent cardiovascular protection, have stimulated substantial interest. Much focus has centered on the vasoactive and angiogenic properties of statins and the possible role of eNOS in mediating such effects[158]. Treatment of $eNOS^{-/-}$ mice with statins did not result in protection from ischemic injury.

Statins have been shown to prevent the down-regulation of eNOS mRNA by ox-LDL[159,160] , hypoxia and TNF-α[150]. Indeed, HMG-CoA reductase inhibition has been associated with increased eNOS expression in human endothelial cells by the prolongation of its already-long mRNA half-life[159,161] . A noted prolongation of eNOS transcript half-life was consistent with the observed 3-fold increase in steady-state eNOS mRNA levels after 24 hours of statin exposure.

VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) :

As the important role of VEGF in blood vessel development, endothelial biology and vascular disease continues to be revealed[162] , the relationship between VEGF and eNOS/NO becomes increasingly defined. For instance, using a murine ischemic limb model, Murohara et al found that VEGF failed to improve angiogenesis in $eNOS^{-/-}$ mice, suggesting that eNOS was a downstream mediator of VEGF-induced angiogenesis[129] . At the molecular level, Bouloumie et al showed that $VEGF^{165}$ increased steady-state eNOS mRNA expression in HUVEC in a time

and concentration-dependent manner[163] . Transcription arrest studies attributed this enhanced expression to a significant increase in eNOS mRNA stability.

HYPOXIA :

Hypoxia has been associated with both the up-regulation and down-regulation of steady-state eNOS mRNA expression. Given, that the body regularly deals with a wide range of oxygen tensions based on the demands of specific organs, it is not surprising that endothelial cells from various vascular beds should respond to hypoxia differently, depending on the specific in vivo and in vitro milieus, as well as the chronicity and severity of the hypoxic exposure[164-168] .

The histone de-acetylase inhibitor, trichostatin A(TSA) is known to inhibit angiogenesis in response to VEGF and hypoxia[169,170]. Recently, in studies designed to elucidate the mechanism of this inhibition, Rossig et al discovered that TSA treatment of HUVEC markedly decreases steady-state levels of eNOS mRNA, without affecting transcription rate as measured by nuclear run-off[171] . TSA treatment increased eNOS promoter–reporter activity. Taken together, these data suggest that TSA modulates eNOS mRNA expression via a post-transcriptional mechanism.

eNOS GENE POLYMORPHISM :

Variants of the NOS3 gene located in the 7q35-q36 region, contains 26 exons with an entire length of 21kb have been investigated for association with hypertension and other cardiovascular disorders [172]. Of these, three polymorphisms have been widely examined for clinical relevance, based on their potential functional effects and their relatively high minor allele frequency in various ethnic groups[172,173]. (Figure 15)

(i) a G894T substitution in exon7 resulting in a Glu to Asp substitution at codon 298 (rs1799983).

(ii) an insertion-deletion in **intron4** (4a/b) consisting of two alleles (the a-deletion which has four tandem 27-bp repeats and the b-insertion having five repeats).

(iii) a T786C substitution in the promoter region (rs2070744).

Several polymorphisms of *eNOS* gene are found to be associated with increased risk for cardiovascular disease. Of these 894 G greater than T variant in exon 7 is reported to be associated with cardiovascular disease [308] while 786 T greater than C polymorphism has been associated with hypertension[310] and with coronary spasm. [311]A 27 bp VNTR located in **intron4 of *eNOS* gene** was proven to be of equal interest. It was demonstrated that **27bp repeat in the *eNOS* gene could bind nuclear proteins as an enhancer/repressor to promote/suppress the transcription efficiency.** Functional significance of this polymorphism was also identified in cases with endothelial dysfunction. Wang *et al*[312] reported a significant association of this intron with CAD. Other studies on *eNOS* intron4 polymorphism showed positive association with renal disease [313,314] , essential hypertension among Japanese [315] and stroke among Chinese [316] . However, studies from Taiwan [317] did not reveal any association with premature CAD. The discrepancy in these studies on the association of *eNOS* intron 4b/a VNTR polymorphism with essential hypertension may be related to ethnic diversity. Hence, we investigated this polymorphism in the patients attending the hypertension clinic to find the extent of risk caused by this gene, if any.

AIM OF THE STUDY

AIMS AND OBJECTIVES

Endothelial dysfunction is considered as the cornerstone in the pathogenesis of essential hypertension. Nitric oxide , the second messenger is found to be the main factor involved in endothelial dysfunction and its level is found to be altered in hypertensive states. As the eNOS is the major enzyme responsible for nitric oxide production, variation in its expression and activity can be linked to hypertension.

In this study, the aim is to elucidate the association between the intron4a/b polymorphism in *NOS3gene* with NO levels and essential hypertension. The specific objectives were :

1. To study the levels of NO in the serum of patients with essential hypertension and controls

2. To determine the genotype frequencies of the eNOS intron4 VNTR polymorphism in the extracted DNA of the patients with essential hypertension and controls.

3. To determine if there is any correlation between these polymorphisms and essential hypertension.

4. To determine if there is any correlation between these polymorphisms and serum NO levels.

MATERIALS & METHODS

MATERIALS AND METHODS

STUDY DESIGN : case- control study.

STUDY POPULATION :

CASES :

The study sample is comprised of 150 unrelated essential hypertensive patients attending the outpatient hypertension clinic in Govt. General Hospital , Chennai, which included 131 males and 19 females, of mean age 50.59 $\pm$ 10.52 years.

Inclusion criteria :

Hypertensives, on treatment for 5 – 10 years.

Exclusion criteria :

1. secondary hypertension
2. diabetes mellitus
3. renal failure
3. fever
5. acute infections
6. chronic inflammatory states
7. drugs – oral contraceptive pills , steroids
8. chronic smokers.

CONTROL SUBJECTS :

130 apparently healthy normotensive controls were taken up from the out-patient department, during their visit for master health check up and minor health ailments. Confounding factors like age, sex, smoking, alcoholism were matched. Exclusion criteria were the same as that of cases.

METHODS:

Resting blood pressure was recorded in each subject, after a thirty minutes rest on a couch. Height and weight were recorded and blood samples were collected by venipuncture after an overnight fasting in two test tubes.

1. 2 mL of sample taken in EDTA-NaF tube was centrifuged at 2000 rpm for twenty minutes. **The buffy coat was used for DNA extraction** and plasma for glucose estimation.

2. 3 mL of blood was taken in a plain tube, centrifuged at 2000 rpm for 20 minutes and serum was placed in two aliquots –

    a. for estimating lipid profile, serum urea & creatinine

    b. for serum NOx estimation.

BIOCHEMICAL MARKERS:

Plasma glucose , serum urea , serum creatintine were estimated. Total cholesterol (TC), high density lipoprotein cholesterol (HDL-c) and triglyceride concentration (TGL) were determined enzymatically using kits and XL-300 auto

analyzer at Centralized Biochemistry Laboratory at G.G.H, Chennai-3. Low density lipoprotein cholesterol (LDL-c) was calculated using Friedwald's formula[304]. Serum NOx estimated by Griess method

DNA EXTRACTION BY MODIFIED HIGH SALT METHOD[305]

RBC Lysis :

- 400µL of buffy coat in a 2mL eppendorf was mixed with 1.6mL of 0.17M ammonium chloride and mixed by inversion for about 10 minutes, until red cells were lysed.
- The cells were then centrifuged at 4000rpm for 10 minutes.
- The white cell pellet was washed with 800µL of 0.17M ammonium chloride solution.The procedure was repeated till a clear white cell pellet was obtained.

WBC Lysis :

- To the pellet 500 µL of TKM I* solution was added. It was centrifuged at 10,000 rpm for 10minutes.

Nuclear Lysis :

- The supernatant was discarded and 500 µL of TKM II* solution was added to the pellet. 300 µL of 6M Nacl and 50 µL of 10% SDS was then added.
- Mixed well in a vortex and centrifuged at 10,000 rpm for 10 minutes.

- The supernatant was transferred to a 1.5mL eppendorf.

* TKM-I solution contains 10mM TRIS HCl,10mM KCl,10mM MgCl2 & 2mM EDTA
TKM-II solution contains 10mM TRIS HCl , 10mM KCl, 10 mM MgCl2, 2mM EDTA and 0.4 M Nacl

DNA Precipitation :

- To the supernatant double the volume of 100% ethanol was added.
- The sample was stored at -20◦C for 1 hour.
- It was then centrifuged at 10,000 rpm for 20minutes at 4◦C in a refrigerated centrifuge.
- The supernatant was discarded and 500 µL of 70% ethanol was added to the pellet. It was mixed and centrifuged at 10,000 rpm for 10 minutes at 4◦C.
- Supernatant was discarded and the pellet was air dried.

Storage :

- To the pellet 30 µL of LTE buffer was added and the extracted DNA was stored at -20◦C for future use.

Identification :

- Extracted DNA was identified following an electrophoretic run using 0.8% agarose with a constant voltage of 7V/ cm along with a comparison with a known molecular weight 1kb DNA ladder. (Figure: 17)

Figure 17 :

GEL DOCUMENTATION OF EXTRACTED DNA :

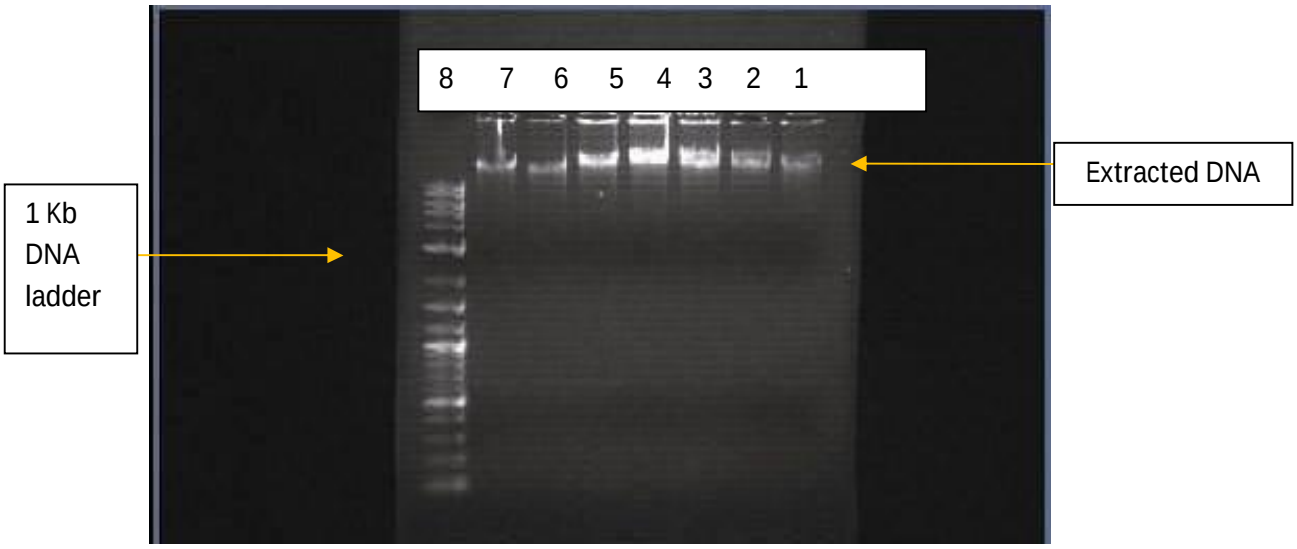
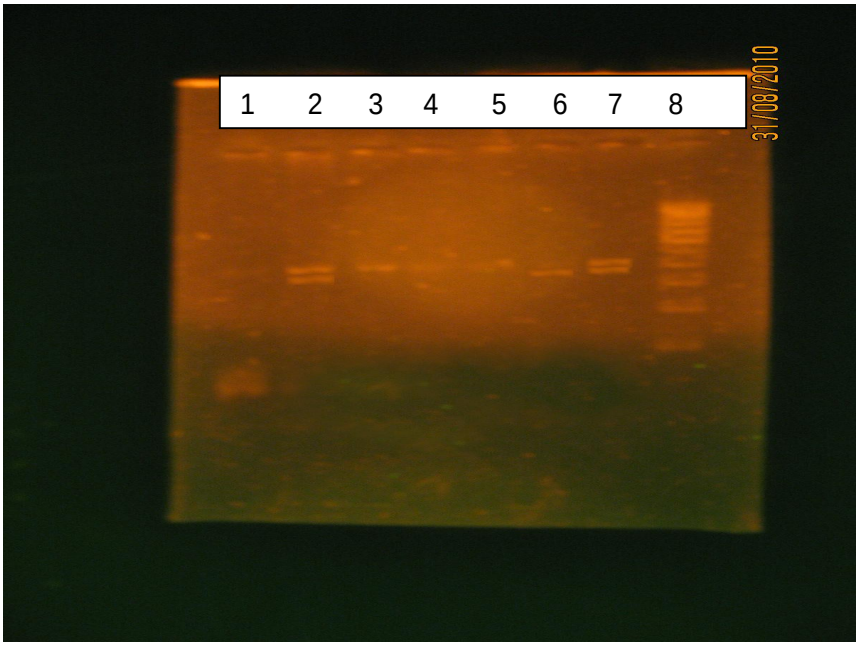


Figure 18 :

ELECTROPHORETOGRAM OF PCR PRODUCTS



Lane 6 - aa genotype

Lane 2,7 – ab genotype

Lane 1,3,4,5 – bb genotype

Lane 8 – 100 bp DNA ladder

POLYMERASE CHAIN REACTION

- eNOS gene was amplified using,
    - Forward primer – 5′-AGGCCCTATGGTAGAGCCTT-3′ and
    - Reverse primer – 5′-TCTCTTAGTGCTGTGGTCAC-3′

PRIMER RECONSTITUTION:

Primers were supplied in lyophilized form. Autoclaved distilled water was used to prepare 100x concentrations i.e., 10 times the molecular weight of primer was the volume of water required to prepare 100x concentrations which was 100 µmolar solution.

- From this stock solution 10x concentration was prepared as the working solution for PCR.

MASTER MIX:

- Master Mix consists of a unique inert red dye in addition to basic components necessary for PCR.
- Red Dye master mix was used in the following composition :
    - Reaction buffer consisted of Tris Hcl -10 mM, at pH- 8.3, KCl – 50 mM.
    - $MgCl_2$ - 1.5 mM acts as catalyst.
    - dNTP’s were used in a concentration of 2.5 mM each.
    - Taq polymerase in a concentration of 1.5 U.

- Primers were used in a concentration of 5 pmol and DNA was used in a concentration of 200 ng.

- PCR was carried out in a reaction volume of 25 µL with the following components;

  - PCR master mix – 12.5 µL
  - Forward primer – 0.9 µL
  - Reverse primer – 0.9 µL
  - DNA – 2 .0µL
  - Distilled water – 8.7 µL
  - Total – 25 µL

- Amplification was carried out in a thermal cycler with the following cycling conditions.

  Initial denaturation – 94^0 C -5min

  30 cycles of :

  - denaturation – 94^0C – 1 min
  - annealing - 54^0C – 1min
  - extension - 72^0C – 1min

  Final extension at 72^0C - 10 min.

- Amplified products viz. 393 bp size for 'a' allele and 420 bp size product for 'b' allele were identified by 3% agarose gel electrophoresis by comparison with a known 100bp DNA ladder. Thus, each DNA sample

revealed one of three possible patterns after electrophoresis viz., a 393 bp band (aa genotype), a 420 bp band (bb genotype) or both 393 and 420 bp bands (ab genotype) (Figure 18).

AGAROSE GEL ELECTROPHORESIS :

- PCR product was run on agarose gel in a 50 mL agarose cast as follows: 1.5g of agarose was weighed and dissolved in 50mL of 1x TAE buffer** with a pH of 8.0.

- It was heated in a microwave oven for 60 secs, cooled and 2.5 µL of ethidium bromide (10mg/moL) was added. It was poured into a cast and allowed to solidify for 15 min, freed of any air bubbles before it was kept in the electrophoresis tank. 8 µL of PCR product was loaded onto wells in a consecutive manner and 4 µL of 100 bp DNA ladder was loaded in the last well as a marker. After a run at 8V/cm for 45min, it was visualized under UV illumination.

** To make 50x TAE buffer (250 ml) pH : 8.3

Triose base : 60.5 gm

Glacial acetic acid : 14.27 mL

0.5 M EDTA : 25 mL {pH adjusted to 8 with 10N NaOH }

Make it up to 250 mL with distilled water

MEASUREMENT OF SERUM NOx:

CADMIUM BASED REDUCTION OF NITRATE TO NITRITE FOLLOWED BY GRIESS METHOD :

Principle :

In this method , cadmium, exposed to copper sulfate solution reduces Cu^{2+} to form a porous metallic copper " coat". This copper facilitates electron transfer from cadmium to nitrate, thereby reducing nitrate to nitrite. Cadmium has a reduction potential of -0.403 V. On the other hand , redox potential of nitrate / nitrite is dependent on pH ranging from 0.94 V in acidic solution to 0.015V in basic solution. Therefore, reduction reaction of nitrate to nitrite by cadmium is thermodynamically favourable.

Nitrite reacts with sulfanilamide in acidic media to form a transient diazonium salt. N-naphthyl- ethylenediamine (NED) converts this salt to a stable azo compound with intense purple color, measured at 540 nm.

Procedure :

Step 1 :De-protein**isation**[306] :

300 µL of serum by adding 250 µL of 75 mmol/L $ZnSO_4$ solution, stirring , and centrifuging at 10 000*g* for 1 minute at room temperature, after which 350 µL of 55 mmol/L NaOH was added. Again , the solution was stirred and centrifuged at 10,000*g* for 3 minutes and the supernatant was recovered (free of turbidity). Then diluted by mixing 750 µL of supernatant with 250 µL of glycine buffer (45 g/L, pH 9.7).

Step 2 : Activation of cadmium :

Cadmium granules were rinsed three times, with deionized distilled water and mixed in a shaker gently in a 200 mmol/L copper sulfate[307] solution in glycine-NaOH buffer (15 g/L, pH 9.7) for 5 minutes till the color of the solution fades. The solution was drained off and the step was repeated. The copper-coated granules dried in tissue paper, should be used within 10 minutes. After use, the granules were rinsed and stored in 100 mmol/L H_2SO_4 solution; they can be regenerated by repeating the steps.

Step 3 : Reduction of nitrate :

The nitrite and nitrate calibrators were diluted with glycine buffer . Calibration curves were made over a linear range of nitrite between 0 and 100 µmol/L. Freshly activated cadmium granules (2–2.5 g) were added to 1 mL of pre-treated de-proteinized serum and calibrator. After continuous stirring for 10 minutes, the samples were transferred to appropriately labeled tubes for nitrite determination.

Step 4 : Nitrite assay :

Nitrite was estimated by Griess method.

Reagent 1 : 50 mg of *N*-naphthylethylenediamine dissolved in 250 mL of distilled water.

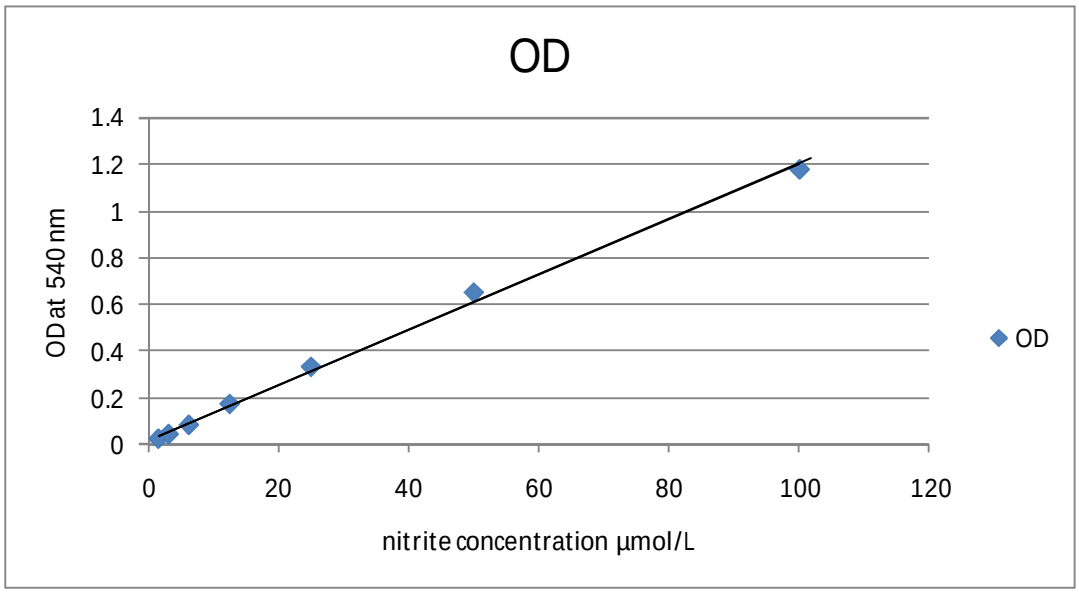
Reagent 2 : 5 g of sulfanilic acid in 500 mL of 3 mol/L HCl.

Both solutions are stable for one year at 4 °C.

From the above tubes 200 µL of sample were placed into fresh glass tubes. Then 800 µL sulfanilic acid solution was mixed, followed by 750 µL NED solution.

Graph : 1

STANDARD CURVE FOR NITRITE LEVEL:



After an incubation period of 10 minutes at room temperature, pink color was developed and its absorbance read at 545 nm within 60 min. The measured OD was plotted on the standardization graph and concentration found out.(Graph : 1)

PLASMA GLUCOSE ESTIMATION

Method : Glucose oxidase- peroxidase method

Principle :

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{glucose oxidase}} \text{gluconic acid} + H_2O_2$$

$$H_2O_2 + \text{phenol} + \text{4 aminoantipyrine} \xrightarrow{\text{peroxidase}} \text{quinoneimine dye} + 2H_2O$$

The concentration of glucose present in the sample, directly propotional to the intensity of pink colour developed , was measured at 540 nm.

Reagents :

Reagent 1 : glucose reagent

Glucose oxidase	20000 IU/L
Peroxidase	3250 IU/L
4-aminoantipyrine	0.52 mmol/L
4-hydroxybenzoic acid	10 mmol/L
Phosphate buffer	110 mmol/L

Standard : glucose 100 mg/dL

Procedure : To 1 mL of reagent, 10 µL sample was added and reading was taken after an incubation period of 15 mins .

Reference value :

Fasting glucose – 70 - 100 mg/dL

Post prandial – upto 140 mg/dL

ESTIMATION OF UREA :

Method : UREASE- GLDH

Principle :

$$\text{Urea} + H_2O \xrightarrow{\text{urease}} 2NH4 + Co2$$

$$NH_4 + \text{NADH} + \text{2-oxoglutarate} \xrightarrow{\text{GLDH}} \text{glutamate} + NAD^+$$

The initial rate of decrease in absorbance at 340nm is propotional to the urea concentration in the sample .

Reagents :

R1 :	α-ketoglutaric acid	99.8 mmml/L
	Urease	23.5 KU/L
	GLDH	3.5 KU/L
	Adenosine diphosphate	7.6 mmol/L
	Sodium azide	0.1%
R2 :	NADH	2.95 mmol/L
	Sodium azide	0.1 %

Working reagent was prepared by mixing R1 & R2 in the ratio of 4:1.

Procedure : To 1 mL of working reagent, 10µL of sample added, and absorbance read at 340 nm after 30 sec and 90 sec.

Reference value: 15 – 40 mg/dL

ESTIMATION OF SERUM CREATININE :

Method: Jaffe's method (initial rate)

Principle :

Creatinine reacts with alkaline picrate to produce an orange- yellow colored creatinine picrate. The intensity of color was directly propotional to the concentration of creatinine, which was measured in a photometric colorimeter at 500-520 nm.

Reagents :

Reagent 1 : Picric acid 25.8 mmol/L

Reagent 2 : Sodium hydroxide 95 mmol/L

Creatinine standard (2 mg/L)

Procedure : To 1 mL of reagent, 100 µL of sample added and the color intensity was measured at 505 nm.

Reference range :

Males : 0.7 – 1.4 mg/dL

Females : 0.6 – 1.2 mg / dL

LIPID PROFILE

The biochemical parameters undertaken for the study were determined using the following methodologies:

ESTIMATION OF PLASMA TOTAL CHOLESTEROL

Method Cholesterol esterase – cholesterol oxidase

Principle

$$\text{Cholesterol ester} + H_2O \xrightarrow{\text{cholesterol esterase}} \text{cholesterol+ fatty acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{cholesterol oxidase}} \text{cholestenone} + H_2O_2$$

$$H_2O_2 + \text{phenol} + \text{4- aminoantipyrine} \xrightarrow{\text{peroxidase}} \text{red quinine} + 4H_2O$$

The concentration of cholesterol in the sample, directly proportional to the intensity of the red complex (red quinone), was measured at 500nm.

REAGENTS

Reagent 1 (enzymes / chromogen)

Cholesterol esterase ≥ 200U/L

Cholesterol oxidase ≥ 250U/L

Peroxidase ≥ 1000 U/L

4- aminoantipyrine 0.5 mmol/L

Reagent 1A (Buffer)

Pipes buffer, pH 6.90 50mmol/L

Phenol 25mmol/L

Sodium cholate 0.5 mmol/L

Standard : Cholesterol 200mg/dL

Procedure : To 1 mL of the reconstituted reagent, 10 µL of plasma is added and reading is taken after 5 mins of incubation at 37^{o} C.

Reference Values : 150 - 200 mg /dL

ESTIMATION OF PLASMA TRIGLYCERIDE

Method : Enzymatic / colorimetric method

Principle

Triglyceride + H_2O $\xrightarrow{\text{lipoprotein lipase}}$ glycerol + fatty acid

Glycerol + ATP $\xrightarrow{\text{glycerol kinase}}$ glycerol -3- phosphate + ADP

Glycerol -3- phosphate $\xrightarrow{\text{glycerol -3- phosphate oxidase}}$ dihydroxy acetone phosphate + H_2O_2

2 H_2O_2 + 4 aminoantipyrine + ADPS $\xrightarrow{\text{peroxidase}}$ red quinone + H_2O

The intensity of purple colored complex formed during the reaction, directly proportional to the triglyceride concentration in the sample, was measured at 546nm.

REAGENTS

Reagent 1 (enzymes / chromogen)

Lipoprotein lipase	≥ 1100U/L
Glycerol kinase	≥ 800U/L
Glycerol -3- phosphate oxidase	≥ 5000 U/L
Peroxidase	≥ 350 U/L
4- aminoantipyrine	0.7 mmol/L

ATP	0.3 mmol/L

Reagent 1A (Buffer)

Pipes buffer. pH	7.50 mmol/L
ADPS	1mmol/L
Magnesium salt	15 mmol/L

Standard : Triglycerides 200mg / dL

Procedure : To 1 mL of the reconstituted reagent, 10 µL of plasma is added and read at 546nm after incubation at 37^{o}C for 5mins.

Reference ranges : less than 150 mg/dL

ESTIMATION OF HDL CHOLESTEROL:

Method :

Immuno-inhibition (system pack)

Principle :

Chylomicrons, VLDL, and LDL fractions in plasma are separated from HDL by immune-inhibition. Anti human β-lipoprotein antibody in reagent 1, binds to lipoproteins (Chylomicrons, VLDL, and LDL) other than HDL. The antigen-antibody complexes formed block enzyme reactions when reagent 2 is added. Cholesterol esterase and cholesterol oxidase in reagent 2 react, only with HDL-C. Hydrogen peroxide produced by enzyme reactions with HDL-C, yields a blue coloured complex upon oxidase condensation with F-DAOS and 4-aminoantipyrine

(4-AA) in the presence of peroxidase . The intensity of the blue color complex formed at 593 nm was proportional to the HDL-C in the sample.

$$\text{LDL, VLDL \& chylomicrons} \xrightarrow{\text{anti-lipoprotein B antibody}} \text{antigen antibody complex}$$

$$\text{HDL-C} + H_2O + O_2 \xrightarrow{\text{CHE \& CO}} \text{4-cholestenone} + \text{fatty acid} + H_2O_2$$

$$H_2O_2 + \text{DAOS} + \text{4AAP} \xrightarrow{\text{peroxidase}} \text{blue colored complex} + 2H_2O$$

REAGENTS :

Reagent 1 :

Goods buffer pH 7.0 ; 30.0mmol/L

4-AAP 0.9mmol/L

POD 2400 U/L

Ascorbate oxidase 2700 U/L

Antihuman β lipoprotein antibody

Reagent 2 :

Goods buffer, pH – 7.0 ; 30.0mmol/L

CHE 4000 U/L

CO 20000 U/L

F-DAOS 0.8 mmol/L

Calibrator : HDL-C 56.5 mg/dL

Procedure : Reagent 1 & 2 were mixed in the ratio of 3:1 (1 bottle of reagent 1, was mixed with 1 bottle of reagent 2) and placed in the auto analyser with the following assay parameters:

Assay type	:	2 point
Primary wavelength nm	:	600, secondary wavelength nm : 700
R-1 volume	:	270, R-2 volume : 90
Reaction direction	:	increasing, sample volume : 3 µL
Calibration	:	straight

Reference Values

Adult male	:	35 – 80 mg /dL
Adult female	:	40.0 – 90.0 mg / dL

VLDL and LDL Cholesterol :

These parameters were calculated using Friedwald's formula given below (not applicable if serum Triglycerides exceeds 400 mg/dL)

LDL-C = TC - (HDL-C+ VLDL-C)

VLDL-C = TGL/5.

RESULTS

&

ANALYSIS

STATISTICAL ANALYSIS

1. Allele frequencies were calculated by allele counting.

2. Age, sex, smoking, alcoholism, BMI, plasma glucose, serum urea, serum creatinine, serum lipid levels were compared between control subjects and patients by students ' t' test and chi-square test (χ^2).

3. Genotype frequency distribution between cases and controls were compared with a χ^2 test for 2*2 contingency table.

4. Serum NOx distribution between cases and controls were compared by student independent t test. $p < 0.05$ was considered significant.

5. Serum NOx level was compared in different genotypes in the study group using one way anova F test.

6. Logistic regression analysis was performed to evaluate the interaction between human eNOS intron4 genotypes and other variables in relation to the prevalence of essential hypertension. Independent variables included in the analysis were age (quantitative), sex (male/female), smoking(yes/no), alcoholism (yes/no) , serum levels of glucose, urea, creatinine, cholesterol, triglycerides , HDL (quantitative). The analysis was executed by SAS Statistical program Version 6.10 for Macintos.

7. The influence of other biochemical parameters on serum NOx level was analysed through Pearson correlation .

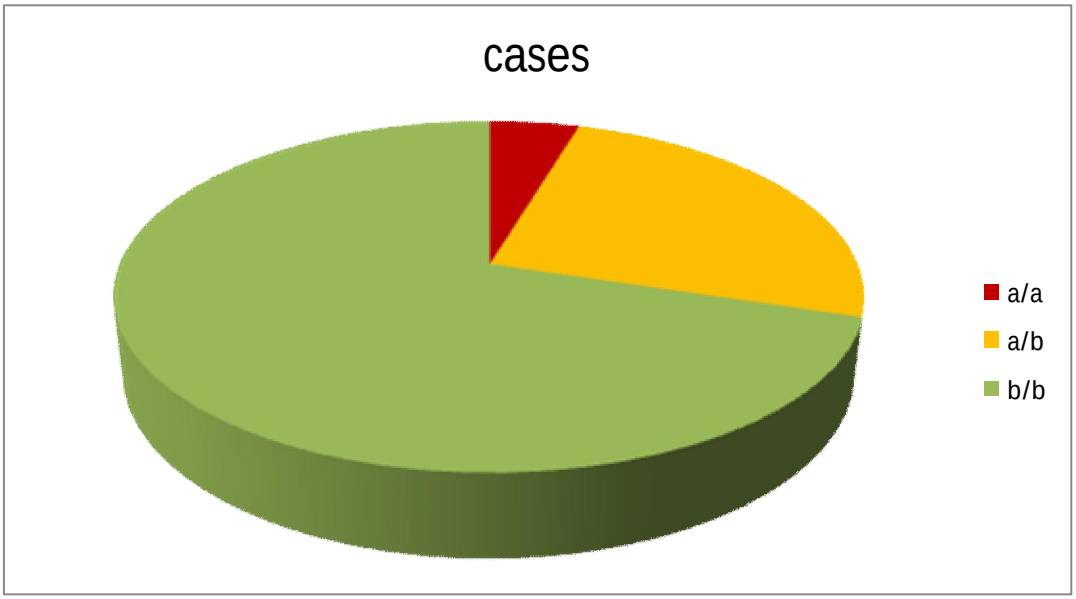
TABLE 3 :

CHARACTERISTICS OF PATIENTS WITH ESSENTIAL HYPERTENSION AND OF CONTROL SUBJECTS.

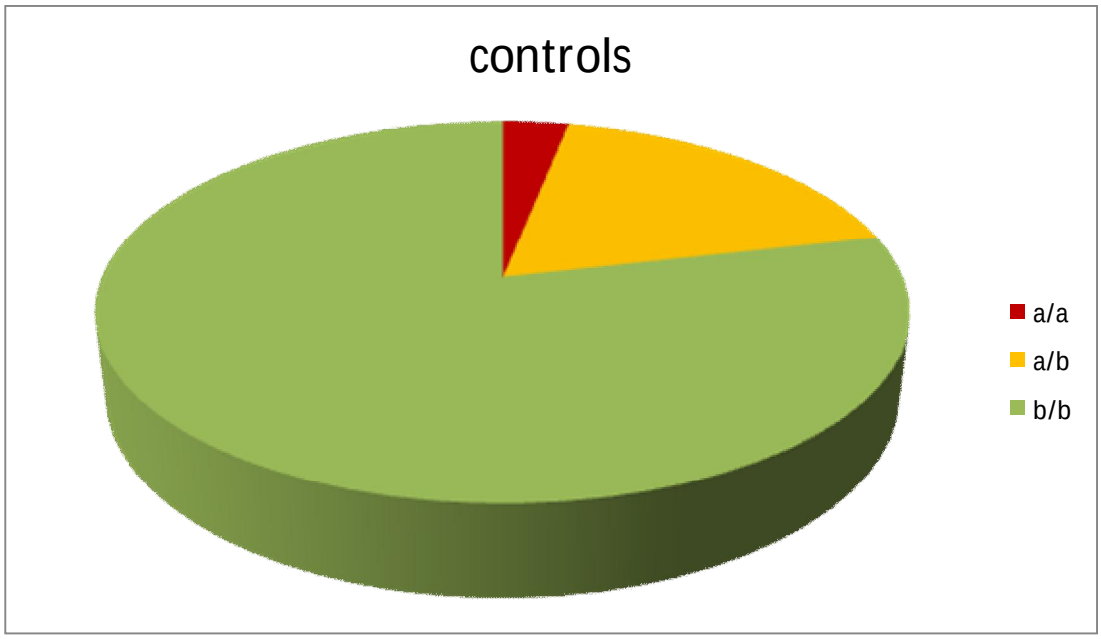
	Group				Student independent t-test
	Hypertensives		Control		
	Mean	SD	Mean	SD	
Age	50.59	10.52	51.83	11.60	t=0.94 P=0.36(NS)
Wt	66.23	9.78	64.42	10.39	t=1.51 P=0.13(NS)
Ht	163.78	6.88	162.15	7.75	t=1.86 P=0.06(NS)
BMI	25.13	3.90	24.47	3.41	t=1.47 P=0.14(NS)

		Group				Pearson Chisquare test
		Hypertensives		Control		
		N	%	n	%	
Sex	Male	132	87.4%	118	90.1%	$\chi 2$=0.49 P=0.48(NS)
	Female	19	12.6%	13	9.9%	
Smoking	No	116	76.8%	96	73.3%	$\chi 2$=0.47 P=0.49(NS)
	Yes	35	23.2%	35	26.7%	
Alcoholism	No	122	81.5%	104	80.2%	$\chi 2$=0.07 P=0.88(NS)
	Yes	28	18.5%	26	19.8%	

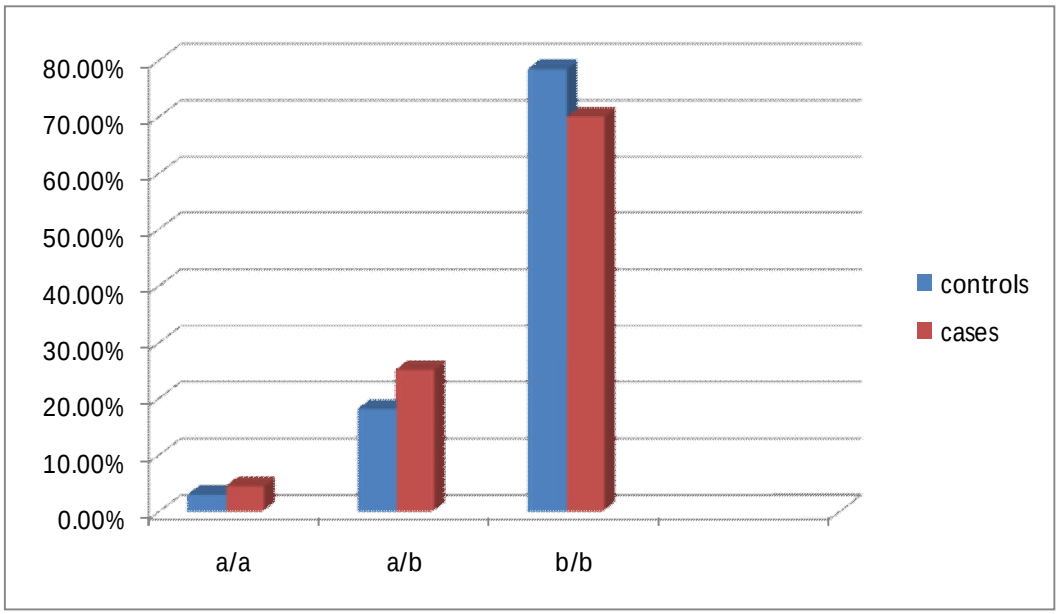
PIE DIAGRAM 1 : GENOTYPE DISTRIBUTION IN HYPERTENSIVES:



PIE DIAGRAM 2 : GENOTYPE DISTRIBUTION IN CONTROLS:

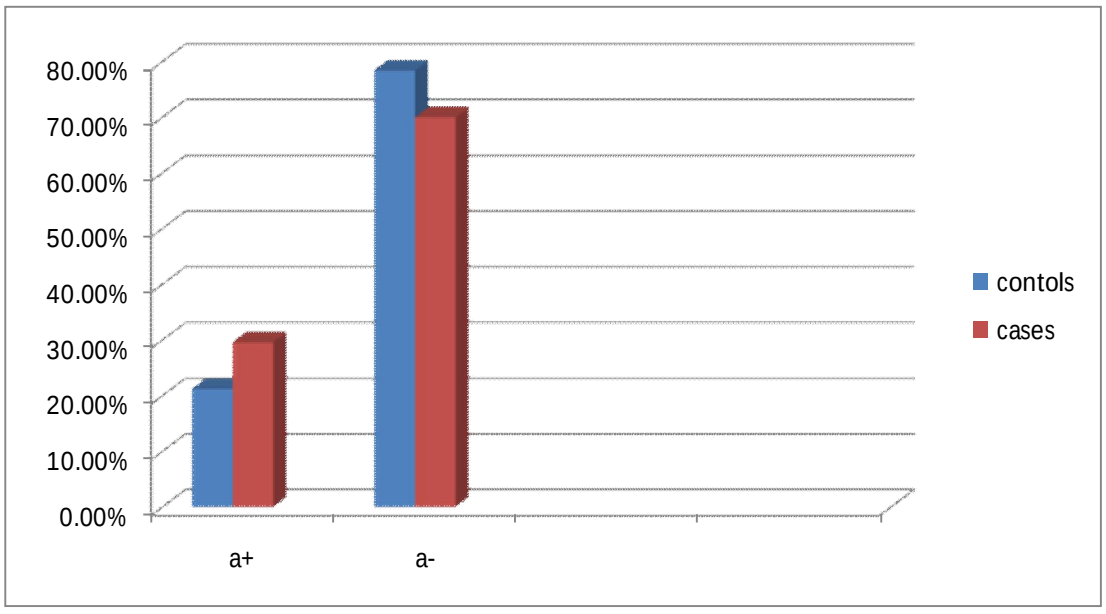


BAR DIAGRAM 1 : COMPARISON OF GENOTYPE DISTRIBUTION BETWEEN CASES AND CONTROLS:



BAR DIAGRAM 2 : GENOTYPE DISTRIBUTION IN CASES AND CONTROLS

.



* a+ represents aa & ab genotypes ; a- represents bb genotype

TABLE 4 :

Genotype distribution and Allele frequencies of human eNOS intron 4 gene

Genotype	Group		Pearson Chisquare test
	Hypertensives	Control	
a/a	7(4.6%)	4(3.1%)	
a/b	38(25.2%)	24(18.3%)	$\chi 2=7.61$ P=0.11
b/b	105(70.2%)	102(78.6%)	
Total	150	130	

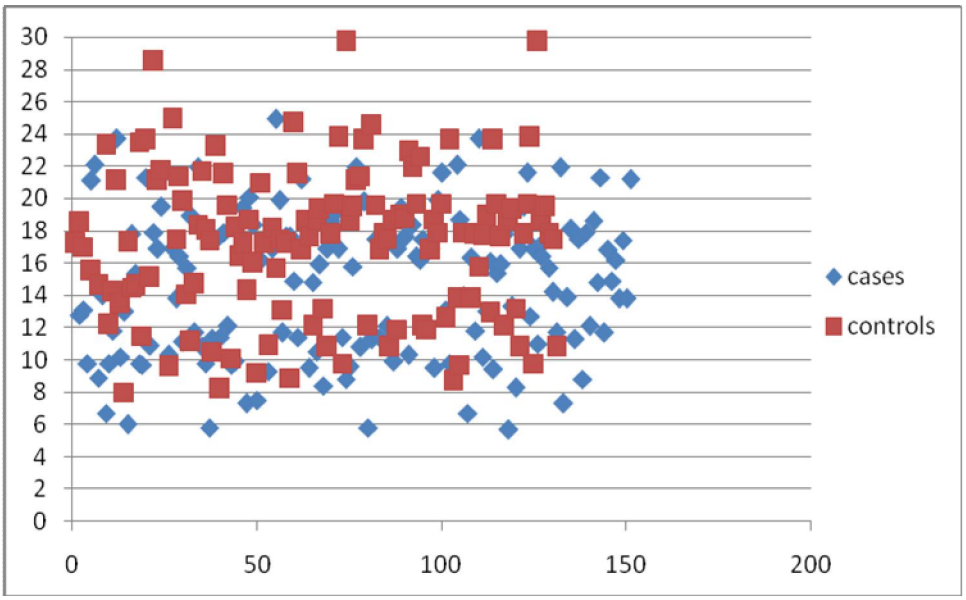
TABLE 5 :

GENOTYPE DISTRIBUTION AND ALLELE FREQUENCIES OF HUMAN eNOS INTRON 4 GENE

Genotype	Hypertensives	Control	P value
a+*	45 (29.8%)	28 (21.4%)	Chi sq = 0.45
a-*	105 (70.2%)	102 (78.6%)	p = 0.5

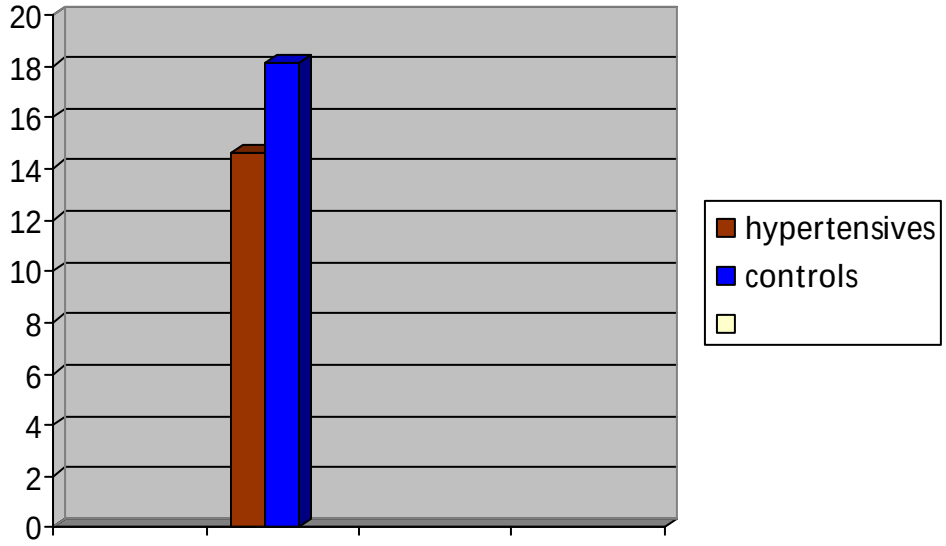
SCATTER DIAGRAM : 1

PATTERN OF DISTRIBUTION OF SERUM NOx LEVEL IN CASES AND CONTROLS



Y axis – serum NOx concentration

BAR DIAGRAM – 3 : COMPARISON OF SERUM NOx LEVEL BETWEEN CASES AND CONTROLS



Y axis – serum NOx concentration in µmol/L

TABLE 6 :

COMPARISION OF SERUM NOx level AMONG CASES AND CONTROLS

	Group				Student independent t-test
	Hypertensives		Control		
	Mean	SD	Mean	SD	
SERUM NOx	14.69	4.45	18.16	7.23	t=4.91 P=0.001***

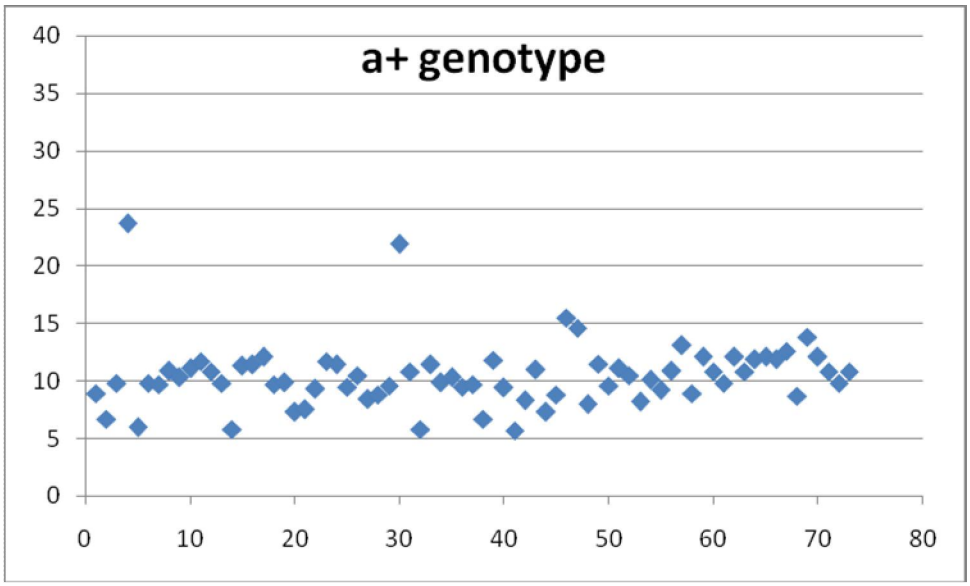
TABLE 7 : SERUM NOx IN VARIOUS GENOTYPES

Variable	a+ Genotype (N=73)	a- Genotype (N=207)	**Student independent t-test**
Serum NOx level	10.45	18.35	t = 11.42 p = 0.001

TABLE 8 : COMPARISON OF SERUM NOx LEVEL BETWEEN INDIVIDUAL GENOTYPES

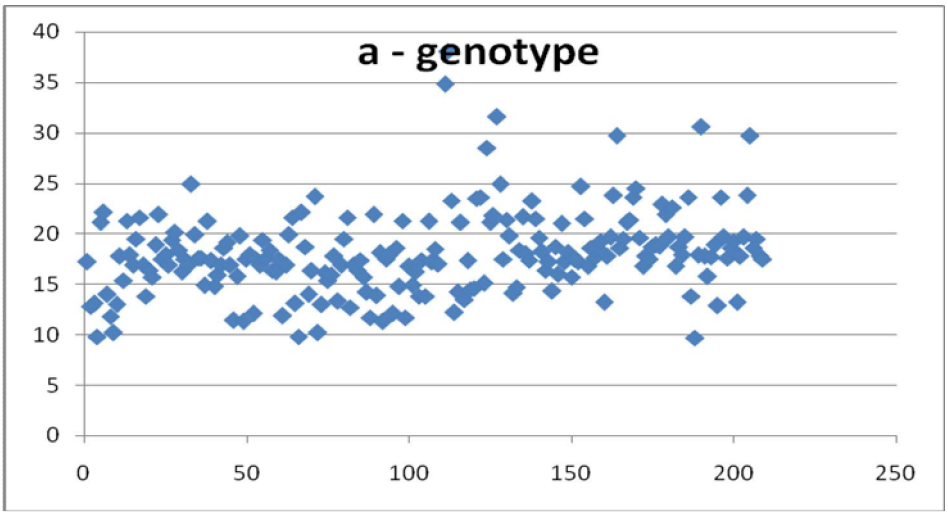
Study groups	Geno types	N	Mean	Std. Deviation	Oneway ANOVA F-test	Post Hoc multiple comparison using Bonferroni t-test
Study	a/a	7	7.457	1.6592	F=70.2 P=0.001	a/a Vs b/b a/b Vs b/b b/b Vs a/a, b/b
	a/b	38	10.513	3.3170		
	b/b	105	16.671	3.2350		
	Total	150	14.694	4.4478		
Control	a/a	4	9.425	1.6761	F=22.6 P=0.001	a/a Vs b/b a/b Vs b/b b/b Vs a/a, b/b
	a/b	24	11.400	1.7245		
	b/b	102	20.074	6.9632		
	Total	130	18.160	7.2332		

SCATTER DIAGRAM 2 : PATTERN OF DISTRIBUTION OF SERUM NOx LEVEL IN a+ GENOTYPIC INDIVIDUALS



Y axis – serum NOx concentration in µmol/L

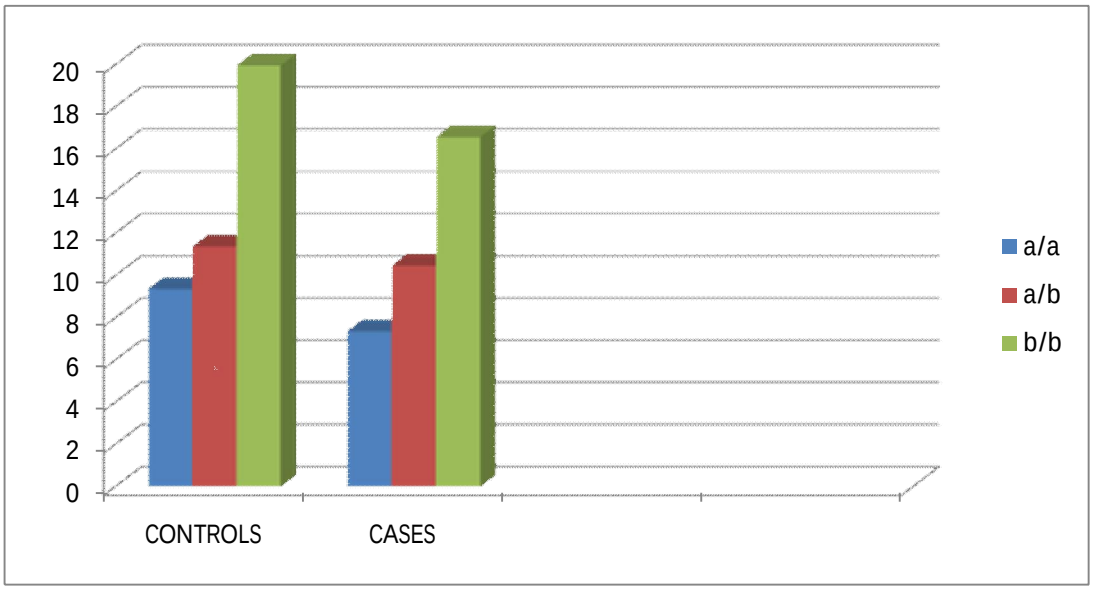
SCATTER DIAGRAM 3 : PATTERN OF DISTRIBUTION OF SERUM NOx LEVEL IN a+ GENOTYPIC INDIVIDUALS



Y axis – serum NOx concentration in µmol/L

BAR DIAGRAM : 4

SERUM NOx LEVEL IN VARIOUS GENOTYPES:



Y axis – serum NOx concentration in µmol/L

TABLE 9 : COMPARISON OF OTHER BIOCHEMICAL PARAMETERS.

	Group				Student independent t-test
	Hypertensives		Control		
	Mean	SD	Mean	SD	
Blood sugar	96.15	6.81	94.69	7.16	t=1.75 P=0.08
S.urea	27.7	5.161	28.1	5.083	t=0.192 p=.848
S.creatinine	.849	.127	.854	0.128	t=.330 p=.742
T.CHOL	162.88	31.13	164.12	42.73	t=0.28 P=0.77
TGL	163.99	48.37	165.09	58.33	t=0.17 P=0.86
HDL	38.04	7.70	44.42	12.00	t=5.38 P=0.001
LDL	96.62	37.44	88.44	37.25	t=1.83 P=0.07

TABLE: 10 MULTIPLE LOGISTIC REGRESSION ANALYSIS

	B	Sig.	Exp(B)	95%CI	
				Lower	Upper
AGE	-.002	.928	.998	.952	1.046
SEX	-.395	.657	.673	.117	3.865
SMOKING	-1.394	.117	.248	.043	1.420
ALCOHOLIC	1.414	.155	4.114	.587	28.846
BMI	.088	.116	1.092	.978	1.220
Bl.SUGAR	.025	.448	1.025	.961	1.093
T.CHOL	-.017	.446	.983	.941	1.027
TGL	.002	.728	1.002	.990	1.015
HDL	.025	.435	1.025	.963	1.090
LDL	.022	.251	1.023	.984	1.062
NOx	.765	.000	2.149	1.759	2.625
Constant	-13.962	.001	.000		

TABLE 11 :

CASE AND CONTROL GROUP CORRELATIONS OF SERUM NOx LEVEL WITH OTHER BIOCHEMICAL PARAMETERS:

	Type of statistical analysis	Plasma glucose	T.chol	TGL	HDL	LDL	SERUM NOx
Serum NOx level	Pearson Correlation	.070	.028	.157	-.015	-.038	1
Of controls	Sig. (2-tailed)	.429	.751	.073	.865	.669	.
	N	130	130	130	130	130	130
Serum NOx	Pearson Correlation	-.026	.094	-.013	.055	.100	1
Of cases	Sig. (2-tailed)	.749	.251	.872	.503	.223	.
	N	150	150	150	150	150	150

** Correlation is significant at the 0.01 level (2-tailed).

RESULTS

- Tables 1 & 2 are the master charts for various data in the study and control groups respectively.

- Table 3 : shows age , sex , BMI and other conventional risk factors distribution in patients and control subjects with an insignificant p value.

- Pie diagrams 1 & 2 show the pattern of distribution of various genotypes in cases and controls respectively.

- Bar diagrams 1 & 2 compares the genotype distribution and allele frequencies of human eNOS intron4 gene in patients with essential hypertension and controls. Homozygous ‘aa’ genotype was found to be more prevalent in the hypertensives (4.6%), when compared to controls (3.1%). In contrast, both homozygous ‘bb’ and heterozygous ‘ab’ were more common in controls when compared with the hypertensives. But these differences were not statistically significant, as represented in tables 4 & 5.

- Scatter diagrams 1 represents the distribution of serum NOx level in cases and controls.

- Table 6 compares serum NOx levels of cases with controls. Statistically significant low NOx levels, with p value of 0.001 was observed in cases (14.69 $\pm$ SD 4.45) when compared to controls (18.16 $\pm$ SD 7.23), as represented in bar diagram 3.

- Scatter diagrams 2 & 3 represents the distribution of serum NOx level in a+ and a- genotypes.

- Bar diagram 4 compares the distribution of serum NOx level in various genotypes.

- Table 7 & 8 shows the comparison of serum NOx between various genotypes in both the groups. The activity was significantly lower among a+ genotype individuals(10.45) when compared to a- genotype individuals (18.35) with a significant p value.

- Table 9 shows the distribution of other biochemical parameters like plasma glucose, serum urea, serum creatinine, lipid profile between hypertensives and controls. HDL levels was found to be significantly lowered in cases.

- Table 10 shows the multiple logistic regression analysis, from which it was found that serum NOx level is an independent risk factor for hypertension.

- Table 11 shows pearson correlation analysis, from which it was found that serum NOx level was not influenced by biochemical parameters like plasma glucose and lipid profile.

DISCUSSION

DISCUSSION

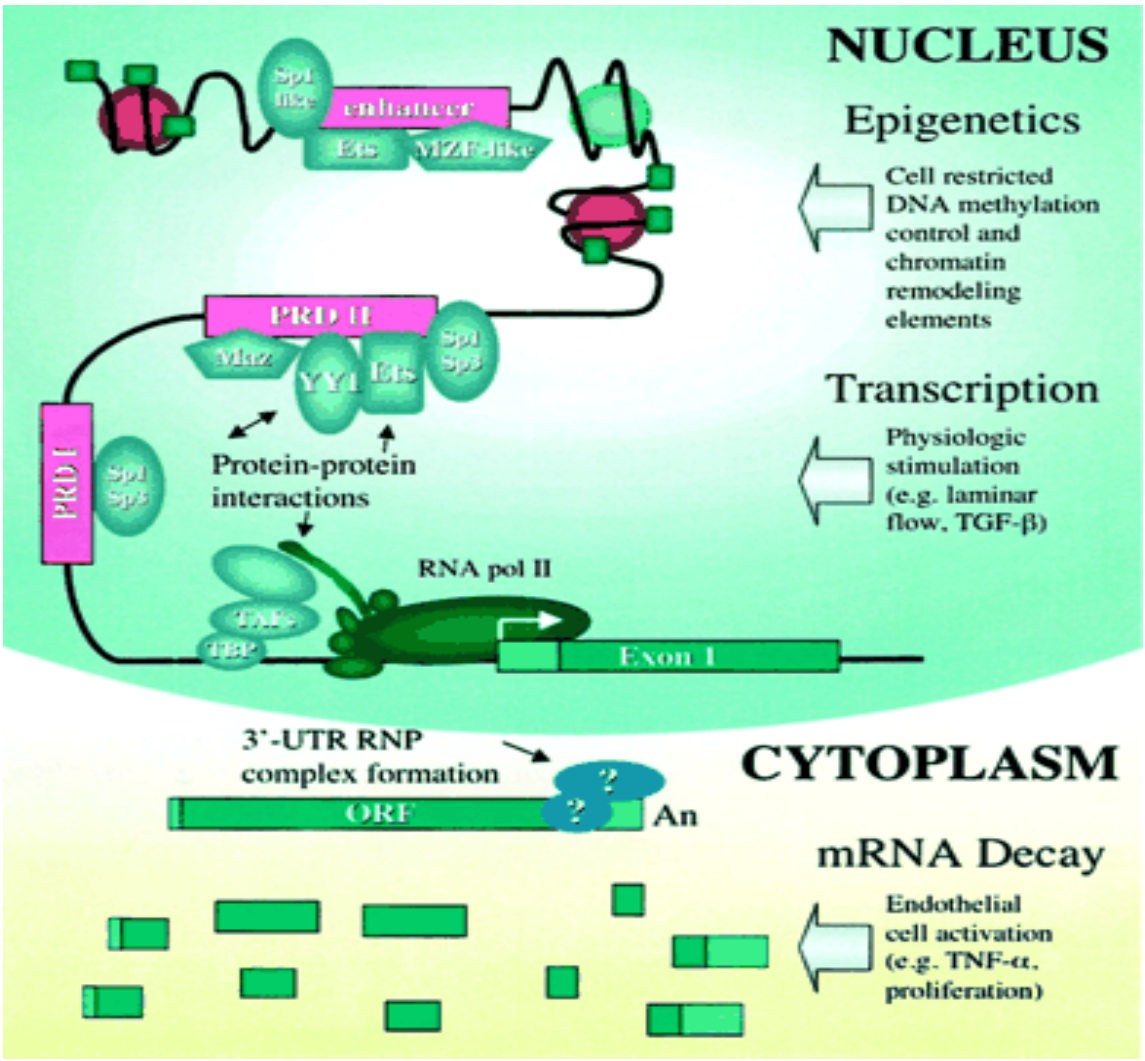
Genetic factors in combination with a number of environmental risk factors are involved in essential hypertension. Endothelial dysfunction which is now recognised to be the hallmark in the pathogenesis of essential hypertension, is mainly caused by imbalance between vasoconstrictors and vasodilators . Nitric oxide, the one considered to be the main factor involved in endothelial dysfunction, is found to be the main mediator involved in shear stress. The effect of physiological vasodilators like acetyl-choline, bradykinin and various therapeutic vasodilators were found to exert their action mainly via NO pathway. The level of NO was found to be altered in various hypertensive disorders . The lowered plasma NO level may be attributed to various factors like reduced biosynthesis and inactivation by free radicals. If it is due to reduced biosynthesis, it may be attributed to polymorphisms at the gene level, reduced transcriptional activity of eNOS gene, inactivation of eNOS and circulating eNOS inhibitors. Various studies are being conducted to find the role of endothelial nitric oxide synthase gene in regulating plasma NO level and in the pathogenesis of essential hypertension. The eNOS gene is regulated by various factors from the level of transcription to mRNA decay as shown in figure 16. Intron4 was found to affect the transcriptional efficiency of the gene and its polymorphism is being studied in relation to essential hypertension.

This study was performed to seek for an apt report to the following queries :

1. Is serum nitric oxide level altered in case of essential hypertension?

Figure : 16

VARIOIUS LEVELS OF REGUALTION OF eNOS GENE.



2. If so, whether the eNOS intron4 gene polymorphism is responsible for any altered effect?

3. Can eNOS intron4 polymorphism be considered as a risk factor for developing essential hypertension ?

In this study, the hypertensives and controls were perfectly matched with respect to the confounding variables like age, sex, BMI, smoking and alcoholism. Those with impaired glucose tolerance, renal failure, acute infections, chronic inflammation and chronic smokers were excluded from the study as these states may present with altered serum NOx level.

The main area of study was focused on serum NO level (NOx index) and eNOS intron4 polymorphism screening (genotypes aa , ab , bb).

On comparing the serum NOx level of the hypertensives with the controls, it was found to be significantly lowered in hypertensives (14.69 versus 18.16, p=0.001). Previous studies (Afrasyap & Ozturk, 2004; Kumar & Das, 2000: Node, K., Kitakaze, M., Yoshikawa, H., Kosaka, H., & Hori, M. (1997)) have shown similar supportive results.Node K et al (1997), in fact recorded a very similar decline in NO level in hypertensives when compared to controls. Different studies showed a wide variation of the normal range of NO (Ferlito, Gallina, Pitari, & Bianchi, 1998; Klahr, 2001; Kumar & Das, 2000; Jeerooburkhan et al., 2001). It is quite obvious that the serum NO level is an independent risk indicator in essential hypertension, based on this study.

In the present study the frequency of ‘a’ allele was found to be approximately 0.26, which is little higher than that found in other populations viz., Iranian (0.1), Japanese (0.1 to 0.13) , Turkish (0.14), Australian (0.17) and little lower than that of African Americans (0.28). The differences in the ethnic origin and sample sizes might have an influence in the results obtained regarding the distribution of the eNOS intron4 polymorphism studied in these populations.

This study did not find any correlation between the presence of the eNOS 4a/b variant and essential hypertension. This may be due to the small sample size. According to the literature survey, only two studies, one on Caucasians in general (Rodríguez-Esparragón, Rodríguez-Pérez, Macías-Reyes, & Alamo-Santana, 2003), and another on the Ukrainian population (Dosenko, Zagoriy, Haytovich, Gordok, & Moibenko, 2005), have shown a correlation between this polymorphism and essential hypertension. No such correlation have been found in other studies (Gouni-Berthold et al., 2005; Miyamoto et al., 1998; Zhao, Su, Chen, Li, & Gu, 2006).

Though intron 4a/b polymorphism of NOS3 had no association with essential hypertension, a trend towards a higher frequency of the ‘a’ allele was apparent in the hypertensives. In other studies conducted in India, the same trend was observed (T Padma et al., 2009 ; Kamna Srivastava et al., 2009). This study needs to be expanded by conducting a large cohort study, to elucidate in depth, a link of any association of essential hypertension with intron4 gene polymorphism.

On comparison of the serum NOx levels between the various genotypes (a+ genotype and a- genotype), there was a significantly lower level among a+ (aa &

ab) genotypic hypertensives and controls with a p value was 0.001. This suggests that a+ genotype is significantly associated with low serum NOx level. Based on the study conducted, it may be considered to be responsible for its effect on hypertension .

CONCLUSION

CONCLUSION

- The low serum NOx level may be an independent risk factor for essential hypertension.
- The eNOS intron4 polymorphism may exert an effect on serum NOx levels, probably by altering the transcriptional efficiency.
- ‘a’ allelic persons were found to have lower level of serum NOx, when compared to ‘b’ alleles.
- eNOS intron4 polymorphism was not found to be a risk factor for essential hypertension in this study.
- The presence of ‘a’ allele makes a person more susceptible to essential hypertension because of its nitric oxide lowering effect. ‘b’ allele was found to be protective.
- As the study population was confined to the hypertensives attending a single govt.general hospital, the expected role of eNOS intron4 polymorphism as a risk factor could not be framed.

Scope for future study

SCOPE FOR FURTHER STUDY

- This study needs to be expanded to cover a larger population, to define the actual role of eNOS intron4 polymorphism in essential hypertension.
- A case-cohort study will probably unravel the involvement of this gene aberration in entire South Indian population, which was the aim before commencing this study.
- Studies relating serum NOx level and antioxidant status have to be explored.
- Studies may be further carried out to find the effect of rapid lifestyle modification on serum NOx level and eNOS polymorphism.
- Various other eNOS genes have to be explored and their association with eNOS activity and essential hypertension to be studied.
- Antihypertensive treatment regimens may be targetted to increase the serum NO level by one of the following methods .
    - to find newer NO donors.
    - to inhibit eNOS inhibitors.
    - to enhance the activity of sGC .
    - to increase the transcriptional activity and to prolong mRNA half-life, at the gene level.
- Studies relating to serum eNOS activity , nitro-tyrosine level and antioxidant status in addition to NOx levels, may be explored, to get a more precise NO level.
- **GENE THERAPY** may be ventured if there is an association of the gene polymorphism with essential hypertension.

BIBLIOGRAPHY

BIBLIOGRAPHY

1. Campanini B: *The World Health Report: Reducing Risks, Promoting Healthy Life*, Geneva, World Health Organization, 2002.
2. Brawnwald's heart disease- A textbook of cardiovasular medicine. 8th edition; 1027-1034
3. Kaplan NM, Opie LH: Controversies in hypertension. *Lancet* 2006; 367:168.
4. Chobanian AV, Bakris GL, Black HR, et al: The Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure: The JNC 7 report. *JAMA* 2003; 289:2560.
5. Winkelmayer WC, Stampfer MJ, Willett WC, Curhan GC: Habitual caffeine intake and the risk of hypertension in women. *JAMA* 2005; 294:2330.
6. Taylor EN, Mount DB, Forman JP, Curhan GC: Association of prevalent hypertension with 24-hour urinary excretion of calcium, citrate, and other factors. *Am J Kidney Dis* 2006; 47:780.
7. Intersalt Cooperative Research Group : Intersalt: An international study of electrolyte excretion and blood pressure. Results for 24-hour urinary sodium and potassium excretion. *BMJ* 1988; 297:319.
8. Luft FC: Mendelian forms of human hypertension and mechanisms of disease. *Clin Med Res* 2003; 1:291.
9. Franklin SS, Pio JR, Wong ND, et al: Predictors of new-onset diastolic and systolic hypertension: The Framingham Heart Study. *Circulation* 2005; 111:1121.
10. Franklin SS: Hypertension in older people: Part 1. *J Clin Hypertens (Greenwich)* 2006; 8:444.
11. McEniery CM, Yasmin , Wallace S, et al: Increased stroke volume and aortic stiffness contribute to isolated systolic hypertension in young adults. *Hypertension* 2005; 46:221-226.
12. Guyenet PG: The sympathetic control of blood pressure. *Nat Rev Neurosci* 2006; 7:335-346.

13. Smith PA, Graham LN, Mackintosh AF, Stoker JB, et al: Relationship between central sympathetic activity and stages of human hypertension. *Am J Hypertens* 2004; 17:217-222.

14. Vloet LC, Pel-Little RE, Jansen PA, Jansen RW: High prevalence of postprandial and orthostatic hypotension among geriatric patients admitted to Dutch hospitals. *J Gerontol A Biol Sci Med Sci* 2005; 60:1271.

15. Esler M, Straznicky N, Eikelis N, et al: Mechanisms of sympathetic activation in obesity-related hypertension. *Hypertension* 2006; 48:787.

16. Baguet JP, Narkiewicz K, Mallion JM: Update on hypertension management: Obstructive sleep apnea and hypertension. *J Hypertens* 2006; 24:205.

17. Yaggi HK, Concato J, Kernan WN, et al: Obstructive sleep apnea as a risk factor for stroke and death. *N Engl J Med* 2005; 353:2034.

18. Keller G, Zimmer G, Mall G, et al: Nephron number in patients with primary hypertension. *N Engl J Med* 2003; 348:101.

19. Reyes AJ, Leary WP: Controversies in cardiology. *Lancet* 2006; 367:1314.

20. Vasan RS, Evans JC, Larson MG, et al: Serum aldosterone and the incidence of hypertension in nonhypertensive persons. *N Engl J Med* 2004; 351:33.

21. Stewart AD, Millasseau SC, Dawes M, et al: Aldosterone and left ventricular hypertrophy in Afro-Caribbean subjects with low renin hypertension. *Am J Hypertens* 2006; 19:19.

22. Schiffrin EL: Effects of aldosterone on the vasculature. *Hypertension* 2006; 47:312.

23. Beevers G, Lip GYH, O'Brien E. ABC of hypertension: the pathophysiology of hypertension. British Medical Journal. 2001;322(7291):912–916.

24. Carretero OA, Oparil S. Essential hypertension. Part I: definition and etiology. Circulation. 2000;101(3):329–335.

25. Luft FC. Twins in cardiovascular genetic research. Hypertension. 2001;37(2):350–356.

26. Moncada S, Herman AG, Higgs EA, Vane JR. Differential formation of prostacyclin (PGX or PGI2) by layers of the arterial wall. An explanation for

the antithrombotic properties of vascular endothelium. Thromb Res 1977;11: 323-44.

27. Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 1980; 288: 373-6.

28. Ignarro LJ, Byrns RE, Buga GM, Wood KS. Endothelium-derived relaxing factor from pulmonary artery and vein possesses pharmacologic and chemical properties identical to those of nitric oxide radical. Circ Res 1987; 61: 866-79.

29. Palmer RM, Ferrige AG, Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature 1987; 327: 524-6.

30. Ludmer PL, Selwyn AP, Shook TL, et al. Paradoxical vasoconstriction induced by acetylcholine in atherosclerotic coronary arteries. N Engl J Med 1986; 315: 1046-51.

31. Celermajer DS, Sorensen KE, Gooch VM, et at. Non-invasive detection of endothelial dysfunction in children and adults at risk of atherosclerosis. Lancet 1992; 340: 1111-5.

32. Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature 1993; 362: 801-9.

33. Choen R. The role of nitric oxide and other endothelium-derived vasoactive substances in vascular disease. Prog Cardiovasc Dis 1995; 38: 105-28.

34. Schwartz SM, Gajdusek EM, Selden SC. Vascular wall growth control: the role of endothelium. Arteriosclerosis 1981; 1: 107-61.

35. Okumura K, Yasue H, Matsuyama K, et al. Effect of acetylcholine on the highly stenotic coronary artery: difference between the constrictor response of the infarct-related coronary artery and that of the noninfarct-related artery. J Am Coll Cardiol 1992; 19: 752-8.

36. Sorensen KE, Celermajer DS, Georgakopoulos D, Hatcher G, Betteridge DJ, Deanfield JE. Impairment of endothelium-dependent dilation is an early event in children with familial hypercholesterolemia and is related to the lipoprotein(a) level. J Clin Invest 1994; 93: 50-5.

37. Celermajer DS, Sorensen KE, Georgakopoulos D, et al. Cigarette smoking is associated with dose-related and potentially reversible impairment of

endothelium-dependent dilatation in healthy young adults. Circulation 1993; 88: 2149-55.

38. Panza JA, Quyyumi AA, Brush JE Jr, Epstein SE. Abnormal endothelium dependent vascular relaxation in patients with essential hypertension. N Engl J Med 1990; 323: 22-7

39. McVeigh GE, Brennan GM, Johnston GD, et al. Impaired endothelium-dependent and independent vasodilation in patients with type 2 (non-insulin-dependent) diabetes mellitus. Diabetologia 1992; 35: 771-6.

40. Clarkson P, Celermajer DS, Powe AJ, Donald AE, Henry RM, Deanfield JE. Endothelium-dependent dilatation is impaired in young healthy subjects with a family history of premature coronary disease. Circulation 1997; 96: 3378-83.

41. Woo KS, Chook P, Lollin YI, et al. Hyperhomocyst(e)inemia is a risk factor for anterial endothelial dysfunction in humans. Circulation 1997; 96: 2542-4.

42. Egashira K, Inou T, Hirooka Y, et al. Effects of age on endothelium-dependent vasodilation of resistance coronary artery by acetylcholine in humans. Circulation 1993; 88: 77-81.

43. Mancini GB, Henry GC, Macaya C, et al. Angiotensin-converting enzyme inhibition with quinapril improves endothelial vasomotor dysfunction in patients with coronary artery disease. The TREND (Trial on Reversing Endothelial Dysfunction) Study. Circulation 1996; 94: 258-65.

44. Anderson TJ, Meredith IT, Yeung AC, Frei B, Selwyn AP, Ganz P. The effect of cholesterol-lowering and antioxidant therapy on endothelium-dependent coronary vasomotion. N Engl J Med 1995; 332: 488-93.

45. Treasure CB, Klein JL, Weintraub WS, et al. Beneficial effects of cholesterol-lowering therapy on the coronary endothelium in patients with coronary artery disease. N Engl J Med 1995; 332: 481-7.

46. Husain S, Andrews NP, Mulcahy D, Panza JA, Quyyumi AA. Aspirin improves endothelial dysfunction in atherosclerosis. Circulation 1998; 97: 716-20.

47. Fishman AP. Endothelium: a distributed organ of diverse capabilities. Ann N Y Acad Sci 1982; 401: 1-8.

48. Machovich R. Choices among the possible reaction routes catalyzed by thrombin. Ann N Y Acad Sci 1986; 485: 170-83.

49. Jaffe EA. Cell biology of endothelial cells. Hum Pathol 1987; 18: 234-9. [Links]

50. Hekman CM, Loskutoff DJ. Fibrinolytic pathways and the endothelium. Semin Thromb Hemost 1987; 13: 514-27.

51. Vanhoutte PM, Rubanyi GM, Miller M, Houstin DS. Modulation of vascular smooth muscle cell contraction by the endothelium. Ann Rev Physiol 1986; 48: 349-80.

52. Stamler JS, Loh E, Roddy MA, Currie KE, Creager MA. Nitric oxide regulates basal systemic and pulmonary vascular resistance in healthy humans. Circulation 1994; 89: 2035-40

53. Arnold WP, Mittal CK, Katsuki S, Murad F. Nitric oxide activates guanylate cyclase and increases guanosine 3': 5'-cyclic monophosphate levels in various tissue preparations. Proc Natl Acad Sci USA 1977; 74: 3203-7.

54. Loscalzo J, Welch G. Nitric oxide and its role in the cardiovascular system. Prog Cardiovasc Dis 1995; 38: 87-104.

55. Weksler BB, Marcus AJ, Jaffe EA. Synthesis of prostaglandin I2 (prostacyclin) by cultured human and bovine endothelial cells. Proc Natl Acad Sci USA 1977; 74: 3922-6.

56. Stamler JS, Vaughan DE, Loscalzo J. Synergistic disaggregation of platelets by tissue-type plasminogen activator, prostaglandin E1, and nitroglycerin. Circ Res 1989; 65: 796-804.

57. FitzGerald GA, Pedersen AK, Patrono C. Analysis of prostacyclin and thromboxane biosynthesis in cardiovascular disease. Circulation 1983; 67: 1174-7.

58. Garland CJ, Plane F, Kemp BK, Cocks TM. Endothelium-dependent hyperpolarization: a role in the control of vascular tone. Trends Pharmacol Sci 1995; 16: 23-30.

59. Haynes WG, Webb DJ. Contribution of endogenous generation of endothelin-1 to basal vascular tone. Lancet 1994; 344: 852-4.

60. Pigazzi A, Heydrick S, Folli F, Benoit S, Michelson A, Loscalzo J. Nitric oxide inhibits thrombin receptor-activating peptide-induced phosphoinositide 3-kinase activity in human platelets. J Biol Chem 1999; 274: 14368-75.

61. Cooke JP, Singer AH, Tsao P, et al. Anti-atherogenic affects of L-arginine in the hypercholesterolemic rabbit. J Clin Invest 1992; 90: 1168-72.

62. De Meyer GRY, Bult H, Üstünes L, et al. Effect of nitric oxide donors on neointima formation and vascular reactivity in the collered carotid artery rabbits. J Cardiovasc Pharmacol 1995; 26: 272-9.

63. Gryglewski RJ, Palmer RM, Moncada S. Superoxide anion is involved in the breakdown of endothelium-derived vascular relaxing factor. Nature 1986; 320: 454-6.

64. Keaney JF Jr, Gaziano JM, Xu A, et al. Dietary antioxidants preserve endothelium-dependent vessel relaxation in cholesterol-fed rabbits. Proc Natl Acad Sci USA 1993; 90: 11880-4.

65. Keaney JF Jr, Xu A, Cunningham DC, Jackson T, Frei B, Vita JA. Dietary probucol preserves endothelial function in cholesterol-fed rabbits by limiting vascular oxidative stress and superoxide production. J Clin Invest 1995; 95: 2520-9.

66. Frei B. Reactive oxygen species and antioxidant vitamins: mechanisms of action. Am J Med 1994; 97: 5s-13s.

67. Frei B, England L, Ames, BN. Ascorbate is an outstanding antioxidant in human blood plasma. Proc Natl Acad Sci USA 1989; 86: 6377-81.

68. Levine GN, Frei B, Koulouris SN, Gerhard MD, Keaney JF Jr, Vita JA. Ascorbic acid reverses endothelial vasomotor dysfunction in patients with coronary artery disease. Circulation 1996; 93: 1107-13.

69. Ting H, Timimi F, Haley E, Roddy M, Ganz P, Creager M. Vitamin C improves endothelium-dependent vasodilation in forearm resistance vessels of humans with hypercholesterolemia. Circulation 1997; 95: 2617-22.

70. Heirtzer T, Hanjörg J, Münzel T. Antioxidant Vitamin C improves endothelial dysfunction in chronic smokers. Circulation 1996; 94: 6-9.

71. Vallance P, Leone A, Calver A, Collier J, Moncada S. Accumulation of an endogenous inhibitor of nitric oxide synthesis in chronic renal failure. Lancet 1992; 339: 572-5.

72. Boger RH, Bode-Boger SM, Szuba A, et al. Asymmetric dimethylarginine (ADMA): A novel risk factor for endothelial dysfunction: its role in hypercholesterolemia. Circulation 1998; 98: 1842-7.

73. Cernacek P, Stewart DJ. Immunoreactive endothelin in human plasma: marked elevations in patients in cardiogenic shock. Biochem Biophys Res Commun 1989; 161: 562-7.

74. Shichiri M, Hirata Y, Ando K, et al. Plasma endothelin levels in hypertension and chronic renal failure. Hypertension 1990; 15: 493-6.

75. Lopez JA, Armstrong ML, Piegors DJ, Heistad DD. Vascular responses to endothelin-1 in atherosclerotic primates. Arteriosclerosis 1990; 10: 1113-8.

76. Paravicini TM, Touyz RM: Redox signaling in hypertension. *Cardiovasc Res* 2006; 71:247.

77. Feig DI, Kang DH, Nakagawa T, et al: Uric Acid and hypertension. *Curr Hypertens Rep* 2006; 8:111.

78. Reyes AJ, Leary WP: Controversies in cardiology. *Lancet* 2006; 367:1314.

79. Feelisch, M. and Stamler, J. S. (eds) (1996) Methods in Nitric Oxide Research, John Wiley, Chichester

80. Packer, L. (ed.) (1996) Nitric Oxide Part B Physiological and Pathological Processes. Methods Enzymol. **269**

81. Packer, L. (ed.) (1996) Nitric Oxide Part A Sources and detection of NO; NO synthase. Methods Enzymol. **268**

82. Titheradge, M. A. (ed.) (1998) Nitric Oxide Protocols. Methods Mol. Biol. **100**

83. McMillan, K. and Masters, B. S. (1995) Prokaryotic expression of the heme- and flavin-binding domains of rat neuronal nitric oxide synthase as distinct polypeptides: identification of the heme-binding proximal thiolate ligand as cysteine-415. Biochemistry **34**, 3686–3693

84. Richards, M. K. and Marletta, M. A. (1994) Characterization of neuronal nitric oxide synthase and a C415H mutant, purified from a baculovirus overexpression system. Biochemistry **33**, 14723–14732

85. Ghosh, D. K. and Stuehr, D. J. (1995) Macrophage NO synthase: characterization of isolated oxygenase and reductase domains reveals a head-to-head subunit interaction. Biochemistry **34**, 801–807

86. Crane, B. R., Arvai, A. S., Ghosh, D. K., Wu, C., Getzoff, E. D., Stuehr, D. J. and Tainer, J. A. (1998) Structure of nitric oxide synthase oxygenase dimer with pterin and substrate. Science **279**, 2121–2126

87. Fischmann, T. O., Hruza, A., Niu, X. D., Fossetta, J. D., Lunn, C. A., Dolphin, E., Prongay, A. J., Reichert, P., Lundell, D. J., Narula, S. K. and Weber, P. C. (1999) Structural characterization of nitric oxide synthase isoforms reveals striking active-site conservation. Nat. Struct. Biol. **6**, 233–242

88. Raman, C. S., Li, H., Martasek, P., Kral, V., Masters, B. S. and Poulos, T. L. (1998) Crystal structure of constitutive endothelial nitric oxide synthase: a paradigm for pterin function involving a novel metal center. Cell (Cambridge, Mass.) **95**, 939–950

89. Crane, B. R., Rosenfeld, R. J., Arvai, A. S., Ghosh, D. K., Ghosh, S., Tainer, J. A., Stuehr, D. J. and Getzoff, E. D. (1999) N-terminal domain swapping and metal ion binding in nitric oxide synthase dimerization. EMBO J. **18**, 6271–6281

90. Bredt, D. S. and Snyder, S. H. (1990) Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. Proc. Natl. Acad. Sci. U.S.A. **87**, 682–685

91. Gachhui, R., Presta, A., Bentley, D. F., Abu-Soud, H. M., McArthur, R., Brudvig, G., Ghosh, D. K. and Stuehr, D. J. (1996) Characterization of the reductase domain of rat neuronal nitric oxide synthase generated in the methylotrophic yeast *Pichia pastoris*. Calmodulin response is complete within the reductase domain itself. J. Biol. Chem. **271**, 20594–20602

92. Gachhui, R., Abu-Soud, H. M., Ghosha, D. K., Presta, A., Blazing, M. A., Mayer, B., George, S. E. and Stuehr, D. J. (1998) Neuronal nitric-oxide synthase interaction with calmodulin-troponin C chimeras. J. Biol. Chem. **273**, 5451–5454

93. Abu-Soud, H. M., Yoho, L. L. and Stuehr, D. J. (1994) Calmodulin controls neuronal nitric-oxide synthase by a dual mechanism. Activation of intra- and interdomain electron transfer. J. Biol. Chem. **269**, 32047–32050

94. Corson, M. A., James, N. L., Latta, S. E., Nerem, R. M., Berk, B. C. and Harrison, D. G. (1996) Phosphorylation of endothelial nitric oxide synthase in response to fluid shear stress. Circ. Res. **79**, 984–991

95. Fisslthaler, B., Dimmeler, S., Hermann, C., Busse, R. and Fleming, I. (2000) Phosphorylation and activation of the endothelial nitric oxide synthase by fluid shear stress. Acta Physiol. Scand. **168**, 81–88

96. Dimmeler, S., Fleming, I., Fisslthaler, B., Hermann, C., Busse, R. and Zeiher, A. M. (1999) Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. Nature (London) **399**, 601–605

97. Fulton, D., Gratton, J. P., McCabe, T. J., Fontana, J., Fujio, Y., Walsh, K., Franke, T. F., Papapetropoulos, A. and Sessa, W. C. (1999) Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. Nature (London) **399**, 597–601

98. McCabe, T. J., Fulton, D., Roman, L. J. and Sessa, W. C. (2000) Enhanced electron flux and reduced calmodulin dissociation may explain "calcium-independent" eNOS activation by phosphorylation. J. Biol. Chem. **275**, 6123–6128

99. Komeima, K., Hayashi, Y., Naito, Y. and Watanabe, Y. (2000) Inhibition of neuronal nitric-oxide synthase by calcium/calmodulin-dependent protein kinase IIalpha through Ser847 phosphorylation in NG108–NG115 neuronal cells. J. Biol. Chem. **275**, 28139–28143

100. Hayashi, Y., Nishio, M., Naito, Y., Yokokura, H., Nimura, Y., Hidaka, H. and Watanabe, Y. (1999) Regulation of neuronal nitric-oxide synthase by calmodulin kinases. J. Biol. Chem. **274**, 20597–20602

101. Nakane, M., Mitchell, J., Forstermann, U. and Murad, F. (1991) Phosphorylation by calcium calmodulin-dependent protein kinase II and protein kinase C modulates the activity of nitric oxide synthase. Biochem. Biophys. Res. Commun. **180**, 1396–1402

102. King, S. M., Barbarese, E., Dillman, J. F., Patel-King, R. S., Carson, J. H. and Pfister, K. K. (1996) Brain cytoplasmic and flagellar outer arm dyneins share a highly conserved Mr 8,000 light chain. J. Biol. Chem. **271**, 19358–19366

103. Garcia-Cardena, G., Fan, R., Shah, V., Sorrentino, R., Cirino, G., Papapetropoulos, A. and Sessa, W. C. (1998) Dynamic activation of endothelial nitric oxide synthase by Hsp90. Nature (London) **392**, 821–824

104. Ratovitski, E. A., Alam, M. R., Quick, R. A., McMillan, A., Bao, C., Kozlovsky, C., Hand, T. A., Johnson, R. C., Mains, R. E., Eipper, B. A. and Lowenstein, C. J. (1999) Kalirin inhibition of inducible nitric-oxide synthase. J. Biol. Chem. **274**, 993–999

105. Michel, T. (1999) Targeting and translocation of endothelial nitric oxide synthase. Braz. J. Med. Biol. Res. **32**, 1361–1366

106. Liu, J., Garcia-Cardena, G. and Sessa, W. C. (1995) Biosynthesis and palmitoylation of endothelial nitric oxide synthase: mutagenesis of palmitoylation sites, cysteines-15 and/or -26, argues against depalmitoylation-induced translocation of the enzyme. Biochemistry **34**, 12333–12340

107. Liu, J., Hughes, T. E. and Sessa, W. C. (1997) The first 35 amino acids and fatty acylation sites determine the molecular targeting of endothelial nitric oxide synthase into the Golgi region of cells: a green fluorescent protein study. J. Cell Biol. **137**, 1525–1535

108. Shaul, P. W., Smart, E. J., Robinson, L. J., German, Z., Yuhanna, I. S., Ying, Y., Anderson, R. G. and Michel, T. (1996) Acylation targets endothelial nitric-oxide synthase to plasmalemmal caveolae. J. Biol. Chem. **271**, 6518–6522

109. Prabhakar, P., Thatte, H. S., Goetz, R. M., Cho, M. R., Golan, D. E. and Michel, T. (1998) Receptor-regulated translocation of endothelial nitric-oxide synthase. J. Biol. Chem. **273**, 27383–27388

110. Garcia-Cardena, G., Oh, P., Liu, J., Schnitzer, J. E. and Sessa, W. C. (1996) Targeting of nitric oxide synthase to endothelial cell caveolae via palmitoylation: implications for nitric oxide signaling. Proc. Natl. Acad. Sci. U.S.A. **93**, 6448–6453

111. Feron, O., Belhassen, L., Kobzik, L., Smith, T. W., Kelly, R. A. and Michel, T. (1996) Endothelial nitric oxide synthase targeting to caveolae. Specific

interactions with caveolin isoforms in cardiac myocytes and endothelial cells. J. Biol. Chem. **271**, 22810–22814

112. Ju, H., Zou, R., Venema, V. J. and Venema, R. C. (1997) Direct interaction of endothelial nitric-oxide synthase and caveolin-1 inhibits synthase activity. J. Biol. Chem. **272**, 18522–18525

113. Michel, J. B., Feron, O., Sacks, D. and Michel, T. (1997) Reciprocal regulation of endothelial nitric-oxide synthase by Ca^{2+}-calmodulin and caveolin. J. Biol. Chem. **272**, 15583–15586

114. Venema, V. J., Ju, H., Zou, R. and Venema, R. C. (1997) Interaction of neuronal nitric-oxide synthase with caveolin-3 in skeletal muscle. Identification of a novel caveolin scaffolding/inhibitory domain. J. Biol. Chem. **272**, 28187–28190

115. Eissa, N. T., Strauss, A. J., Haggerty, C. M., Choo, E. K., Chu, S. C. and Moss, J. (1996) Alternative splicing of human inducible nitric-oxide synthase mRNA. Tissue-specific regulation and induction by cytokines. J. Biol. Chem. **271**, 27184–27187

116. Bates, T. E., Loesch, A., Burnstock, G. and Clark, J. B. (1995) Immunocytochemical evidence for a mitochondrially located nitric oxide synthase in brain and liver. Biochem. Biophys. Res. Commun. **213**, 896–900

117. Bates, T. E., Loesch, A., Burnstock, G. and Clark, J. B. (1996) Mitochondrial nitric oxide synthase: a ubiquitous regulator of oxidative phosphorylation?. Biochem. Biophys. Res. Commun. **218**, 40–44

118. Kobzik, L., Stringer, B., Balligand, J.-L., Reid, M. B. and Stamler, J. S. (1995) Endothelial type nitric oxide synthase in skeletal muscle fibers: mitochondrial relationships. Biochem. Biophys. Res. Commun. **211**, 375–381

119. Garvey, E. P., Oplinger, J. A., Furfine, E. S., Kiff, R. J., Laszlo, F., Whittle, B. J. R. and Knowles, R. G. (1997) 1400W is a slow, tight binding and highly selective inhibitor of inducible nitric-oxide synthase *in vitro* and *in vivo*. J. Biol. Chem. **272**, 4959–4963

120. Knowles, R., Dawson, J., Waslidge, N., Russell, R., Angell, A., Craig, C., Schwartz, S., Evans, S., Whittle, B., Garvey, E. (2000) GW273629 is a highly-selective, short-acting iNOS (NOS-2) inhibitor both *in vitro* and *in vivo*: beneficial effects in endotoxin shock. In Biology of Nitric Oxide, vol. 7

(Moncada, S., Gustaffson, L. E., Wiklund, N. P. and Higgs, E. A., eds.), pp. 21, Portland Press, London

121. Bryk, R. and Wolff, D. J. (1998) Mechanism of inducible nitric oxide synthase inactivation by aminoguanidine and L-N^6-(1-iminoethyl)lysine. Biochemistry **37**, 4844–4852

122. Hagen, T. J., Bergmanis, A. A., Kramer, S. W., Fok, K. F., Schmelzer, A. E., Pitzele, B. S., Swenton, L., Jerome, G. M., Kornmeier, C. M., Moore, W. M. (1998) 2-Iminopyrrolidines as potent and selective inhibitors of human inducible nitric oxide synthase. J. Med. Chem. **41**, 3675–3683

123. Hansen, D. W., Peterson, K. B., Trivedi, M., Kramer, S. W., Webber, R. K., Tjoeng, F. S., Moore, W. M., Jerome, G. M., Kornmeier, C. M., Manning, P. T. (1998) 2-Iminohomopiperidinium salts as selective inhibitors of inducible nitric oxide synthase (iNOS). J. Med. Chem. **41**, 1361–1366

124. Huang PL, Huang Z, Mashimo H, Bloch KD, Moskowitz MA, Bevan JA, Fishman MC. Hypertension in mice lacking the gene for endothelial nitric oxide synthase. *Nature*. 1995; 377: 239–242

125. Shesely EG, Maeda N, Kim HS, Desai KM, Krege JH, Laubach VE, Sherman PA, Sessa WC, Smithies O. Elevated blood pressures in mice lacking endothelial nitric oxide synthase. *Proc Natl Acad Sci U S A*. 1996; 93: 13176–13181.

126. Fagan KA, Tyler RC, Sato K, Fouty BW, Morris KG Jr, Huang PL, McMurtry IF, Rodman DM. Relative contributions of endothelial, inducible, and neuronal NOS to tone in the murine pulmonary circulation. *Am J Physiol*. 1999; 277: L472–L478.

127. Rudic RD, Shesely EG, Maeda N, Smithies O, Segal SS, Sessa WC. Direct evidence for the importance of endothelium-derived nitric oxide in vascular remodeling. *J Clin Invest*. 1998; 101: 731–736

128. . Quinlan TR, Li D, Laubach VE, Shesely EG, Zhou N, Johns RA. eNOS-deficient mice show reduced pulmonary vascular proliferation and remodeling to chronic hypoxia. *Am J Physiol Lung Cell Mol Physiol*. 2000; 279: L641–L650.

129. Murohara T, Asahara T, Silver M, Bauters C, Masuda H, Kalka C, Kearney M, Chen D, Symes JF, Fishman MC, Huang PL, Isner JM. Nitric oxide synthase

modulates angiogenesis in response to tissue ischemia. *J Clin Invest.* 1998; 101: 2567–2578.

130. Lee PC, Salyapongse AN, Bragdon GA, Shears LL II, Watkins SC, Edington HD, Billiar TR. Impaired wound healing and angiogenesis in eNOS-deficient mice. *Am J Physiol.* 1999; 277: H1600–H1608.

131. Wilcox JN, Subramanian RR, Sundell CL, Tracey WR, Pollock JS, Harrison DG, Marsden PA. Expression of multiple isoforms of nitric oxide synthase in normal and atherosclerotic vessels. *Arterioscler Thromb Vasc Biol.* 1997; 17: 2479–2488.

132. Fukuchi M, Giaid A. Endothelial expression of endothelial nitric oxide synthase and endothelin-1 in human coronary artery disease. Specific reference to underlying lesion. *Lab Invest.* 1999; 79: 659–670.

133. . Bossaller C, Habib GB, Yamamoto H, Williams C, Wells S, Henry PD. Impaired muscarinic endothelium-dependent relaxation and cyclic guanosine 5'-monophosphate formation in atherosclerotic human coronary artery and rabbit aorta. *J Clin Invest.* 1987; 79: 170–174.

134. Forstermann U, Boissel JP, Kleinert H. Expressional control of the 'constitutive' isoforms of nitric oxide synthase (NOS I and NOS III). *Faseb J.* 1998; 12: 773–790.

135. Marsden PA, Heng HH, Scherer SW, Stewart RJ, Hall AV, Shi XM, Tsui LC, Schappert KT. Structure and chromosomal localization of the human constitutive endothelial nitric oxide synthase gene. *J Biol Chem.* 1993; 268: 17478–17488.

136. Karantzoulis-Fegaras F, Antoniou H, Lai S, Kulkarni G, D'Abreo C, Wong GKT, Miller TL, Chan Y, Atkins J, Wang Y, Marsden PA. Characterization of the human endothelial nitric oxide synthase promoter. *J Biol Chem.* 1999; 274: 3076–3093.

137. Teichert A-M, Karantzoulis-Fegaras F, Chan Y, Marsden P. Cell-specific regulation of the human eNOS gene: a unique model [abstract]. *Acta Physiologica Scandinavica.* 1999; 167: 75

138. Robertson KD, Wolffe AP. DNA methylation in health and disease. *Nature Reviews Genetics.* 2000; 1: 11–19.

139. Traub O, Berk BC. Laminar shear stress: mechanisms by which endothelial cells transduce an atheroprotective force. *Arterioscler Thromb Vasc Biol.* 1998; 18: 677–685.

140. . Sessa WC, Pritchard K, Seyedi N, Wang J, Hintze TH. Chronic exercise in dogs increases coronary vascular nitric oxide production and endothelial cell nitric oxide synthase gene expression. *Circ Res.* 1994; 74: 349–353.

141. . Nadaud S, Philippe M, Arnal JF, Michel JB, Soubrier F. Sustained increase in aortic endothelial nitric oxide synthase expression in vivo in a model of chronic high blood flow. *Circ Res.* 1996; 79: 857–863.

142. Uematsu M, Ohara Y, Navas JP, Nishida K, Murphy TJ, Alexander RW, Nerem RM, Harrison DG. Regulation of endothelial cell nitric oxide synthase mRNA expression by shear stress. *Am J Physiol.* 1995; 269: C1371–C1378.

143. Pintavorn P, Ballermann BJ. TGF-beta and the endothelium during immune injury. *Kidney Int.* 1997; 51: 1401–1412.

144. . Pepper MS. Transforming growth factor-beta: vasculogenesis, angiogenesis, and vessel wall integrity. *Cytokine Growth Factor Rev.* 1997; 8: 21–43.

145. . Inoue N, Venema RC, Sayegh HS, Ohara Y, Murphy TJ, Harrison DG. Molecular regulation of the bovine endothelial cell nitric oxide synthase by transforming growth factor-beta 1. *Arterioscler Thromb Vasc Biol.* 1995; 15: 1255–1261.

146. Saura M, Zaragoza C, Cao W, Bao C, Rodriguez-Puyol M, Rodriguez-Puyol D, Lowenstein CJ. Smad2 mediates transforming growth factor-beta induction of endothelial nitric oxide synthase expression. *Circ Res.* 2002; 91: 806–813.

147. Lopez-Ongil S, Hernandez-Perera O, Navarro-Antolin J, Perez de Lema G, Rodriguez-Puyol M, Lamas S, Rodriguez-Puyol D. Role of reactive oxygen species in the signalling cascade of cyclosporine A-mediated up-regulation of eNOS in vascular endothelial cells. *Br J Pharmacol.* 1998; 124: 447–454.

148. . Navarro-Antolin J, Rey-Campos J, Lamas S. Transcriptional induction of endothelial nitric oxide gene by cyclosporine A. A role for activator protein-1. *J Biol Chem.* 2000; 275: 3075–3080

149. Yoshizumi M, Perrella MA, Burnett JC, Jr., Lee ME. Tumor necrosis factor downregulates an endothelial nitric oxide synthase mRNA by shortening its half-life. *Circ Res.* 1993; 73: 205–209.

150. Laufs U, Fata VL, Liao JK. Inhibition of 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase blocks hypoxia-mediated down-regulation of endothelial nitric oxide synthase. *J Biol Chem.* 1997; 272: 31725–31729.

151. Marsden PA, Schappert KT, Chen HS, Flowers M, Sundell CL, Wilcox JN, Lamas S, Michel T. Molecular cloning and characterization of human endothelial nitric oxide synthase. *FEBS Lett.* 1992; 307: 287–293.

152. Aoki N, Siegfried M, Lefer AM. Anti-EDRF effect of tumor necrosis factor in isolated, perfused cat carotid arteries. *Am J Physiol.* 1989; 256: H1509–H1512.

153. Wang P, Ba ZF, Chaudry IH. Administration of tumor necrosis factor-alpha in vivo depresses endothelium-dependent relaxation. *Am J Physiol.* 1994; 266: H2535–H2541.

154. . Rosenkranz-Weiss P, Sessa WC, Milstien S, Kaufman S, Watson CA, Pober JS. Regulation of nitric oxide synthesis by proinflammatory cytokines in human umbilical vein endothelial cells. Elevations in tetrahydrobiopterin levels enhance endothelial nitric oxide synthase specific activity. *J Clin Invest.* 1994; 93: 2236–2243.

155. Liao JK, Shin WS, Lee WY, Clark SL. Oxidized low-density lipoprotein decreases the expression of endothelial nitric oxide synthase. *J Biol Chem.* 1995; 270: 319–324.

156. Hirata K, Miki N, Kuroda Y, Sakoda T, Kawashima S, Yokoyama M. Low concentration of oxidized low-density lipoprotein and lysophosphatidylcholine upregulate constitutive nitric oxide synthase mRNA expression in bovine aortic endothelial cells. *Circ Res.* 1995; 76: 958–962.

157. Vidal F, Colome C, Martinez-Gonzalez J, Badimon L. Atherogenic concentrations of native low-density lipoproteins down- regulate nitric-oxide-synthase mRNA and protein levels in endothelial cells. *Eur J Biochem.* 1998; 252: 378–384.

158. Endres M, Laufs U, Huang Z, Nakamura T, Huang P, Moskowitz MA, Liao JK. Stroke protection by 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitors mediated by endothelial nitric oxide synthase. *Proc Natl Acad Sci U S A.* 1998; 95: 8880–8885

159. Laufs U, La Fata V, Plutzky J, Liao JK. Upregulation of endothelial nitric oxide synthase by HMG CoA reductase inhibitors. *Circulation.* 1998; 97: 1129–1135.

160. Hernandez-Perera O, Perez-Sala D, Navarro-Antolin J, Sanchez-Pascuala R, Hernandez G, Diaz C, Lamas S. Effects of the 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors, atorvastatin and simvastatin, on the expression of endothelin-1 and endothelial nitric oxide synthase in vascular endothelial cells. *J Clin Invest.* 1998; 101: 2711–2719.

161. Laufs U, Liao JK. Post-transcriptional regulation of endothelial nitric oxide synthase mRNA stability by Rho GTPase. *J Biol Chem.* 1998; 273: 24266–24271.

162. S, Zachary I, Martin JF. VEGF modulates NO production: the basis of a cytoprotective effect? *Cardiovasc Res.* 1999; 41: 509–510.

163. Bouloumie A, Schini-Kerth VB, Busse R. Vascular endothelial growth factor up-regulates nitric oxide synthase expression in endothelial cells. *Cardiovasc Res.* 1999; 41: 773–780.

164. Shaul PW, Wells LB, Horning KM. Acute and prolonged hypoxia attenuate endothelial nitric oxide production in rat pulmonary arteries by different mechanisms. *J Cardiovasc Pharmacol.* 1993; 22: 819–827.

165. Ziesche R, Petkov V, Williams J, Zakeri SM, Mosgoller W, Knofler M, Block LH. Lipopolysaccharide and interleukin 1 augment the effects of hypoxia and inflammation in human pulmonary arterial tissue. *Proc Natl Acad Sci U S A.* 1996; 93: 12478–12483.

166. Justice JM, Tanner MA, Myers PR. Endothelial cell regulation of nitric oxide production during hypoxia in coronary microvessels and epicardial arteries. *J Cell Physiol.* 2000; 182: 359–365.

167. Resta TC, Chicoine LG, Omdahl JL, Walker BR. Maintained upregulation of pulmonary eNOS gene and protein expression during recovery from chronic hypoxia. *Am J Physiol.* 1999; 276: H699–H708.

168. Le Cras TD, Tyler RC, Horan MP, Morris KG, Tuder RM, McMurtry IF, Johns RA, Abman SH. Effects of chronic hypoxia and altered hemodynamics on endothelial nitric oxide synthase expression in the adult rat lung. *J Clin Invest.* 1998; 101: 795–801.

169. Kim MS, Kwon HJ, Lee YM, Baek JH, Jang JE, Lee SW, Moon EJ, Kim HS, Lee SK, Chung HY, Kim CW, Kim KW. Histone deacetylases induce angiogenesis by negative regulation of tumor suppressor genes. *Nat Med.* 2001; 7: 437–443.

170. Deroanne CF, Bonjean K, Servotte S, Devy L, Colige A, Clausse N, Blacher S, Verdin E, Foidart JM, Nusgens BV, Castronovo V. Histone deacetylases inhibitors as anti-angiogenic agents altering vascular endothelial growth factor signaling. *Oncogene.* 2002; 21: 427–436.

171. Rossig L, Li H, Fisslthaler B, Urbich C, Fleming I, Forstermann U, Zeiher AM, Dimmeler S. Inhibitors of histone deacetylation downregulate the expression of endothelial nitric oxide synthase and compromise endothelial cell function in vasorelaxation and angiogenesis. *Circ Res.* 2002; 91: 837–844.

172. Casas JP, Cavalleri GL, Bautista LE, Smeeth L, Humphries SE, Hingorani AD. Endothelial nitric oxide synthase gene polymorphisms and cardiovascular disease: a HuGE review. American Journal of Epidemiology. 2006;164(10):921–935.

173. Cooke GE, Doshi A, Binkley PF. Endothelial nitric oxide synthase gene: prospects for treatment of heart disease. Pharmacogenomics. 2007;8(12):1723–1734.

174. Zintzaras E, Kitsios G, Stefanidis I. Endothelial NO synthase gene polymorphisms and hypertension: a meta-analysis. Hypertension. 2006;48(4):700–710.

175. Wang HG, Wang JL, Chang P, Cao FL, Liu XC, Ma YB, Zhai GX, Gao HQ. Endothelial nitric oxide synthase gene polymorphisms and essential hypertension in Han Chinese.Genet Mol Res. 2010 ;9(3):1896-907.

176. Zintzaras E, Kitsios G, Stefanidis L. Endothelial NO Synthase Gene Polymorphisms and Hypertension . A Meta-Analysis . Hypertension. 2006;48:700.

177. Thomas GD, Zhang W, Victor RG. Nitric oxide deficiency as a cause of clinical hypertension: promising new drug targets for refractory hypertension. JAMA. 2001; 285: 2055–2057.

178. Georgios D, Kitsios F, Elias Zintzaras . . An NOS3 Haplotype is Protective against Hypertension in a Caucasian Population.Int J Hypertens. 2010; 2010: 865031.

179. **Nitric oxide**. Part A. Sources and detection of **NO**; **NO** synthase. Methods Enzymol 1996;268:3–515..

180. Malinski T, Taha Z. **Nitric oxide** release from a single cell measured in situ by a porphyrinic-based microsensor. Nature 1992;358:676-678.

181. Vallance P, Patton S, Bhagat K, MacAllister R, R**ado**mski M, Moncada S, Malinski T. Direct measurement of **nitric oxide** in human beings. Lancet 1995;346:153-154

182. Archer S. Measurement of **nitric oxide** in biological models. FASEB J 1993;7:349-360.

183. Murphy ME, **No**ack E. **Nitric oxide** assay using hemoglobin method. Methods Enzymol 1994;233:240-250.

184. Moshage H, Kok B, Huizenga JR, Jansen PLM. Nitrite and nitrate determinations in plasma: a critical evaluation. Clin Chem 1995;41:892-896.

185. Bories PN, Bories C. Nitrate determination in biological fluids by an enzymatic one-step assay with nitrate reductase. Clin Chem 1995;41:904-907.

186. Green LC, Wagner DA, Glogowski J, Skipper PL, Wish**no**k JS, Tannenbaum SR. Analysis of nitrate, nitrite and [^{15}N]nitrate in biological fluids. Anal Biochem 1982;126:131-138.

187. Kemperers AJ, Luft AG. Re-examination of the determination of environmental nitrate as nitrite by reduction with hydrazine. Analyst 1988;113:1117-1120

188. Muscara MN, de Nucci G. Simultaneous determination of nitrite and nitrate anions in plasma, urine and cell culture supernatants by high-performance liquid chromatography with post-column reactions. J Chromatogr B 1996;686:157-164.

189. Ueda T, Maekawa T, Sadamitsu D, Oshita S, Ogi**no** K, Nakamura K. The determination of nitrite and nitrate in human blood plasma by capillary zone electrophoresis. Electrophoresis 1995;16:1002-1004.

190. Leone AM, Francis PL, Rhodes P, Moncada S. A rapid and simple method for the measurement of nitrite and nitrate in plasma by high performance capillary electrophoresis. Biochem Biophys Res Commun 1994;200:951-957.

191. Everett SA, Dennis MF, Tozer GM, Prise VE, Wardman P, Stratford MRL. **Nitric oxide** in biological fluids: analysis of nitrite and nitrate by high-performance ion chromatography. J Chromatogr A 1995;706:437-442.

192. Yang F, Troncy E, Francoeur M, Vinet B, Vinay P, Czaika G, Blaise G. Effect of reducing reagents and temperatures on conversion of nitrite and nitrate to **nitric oxide** and detection of **NO** by chemiluminescence. Clin Chem 1997;43:657-662.

193. Rhodes PM, Leone AM, Francis PL, Struthers AD, Moncada S. The L-arginine:**nitric oxide** pathway is the major source of plasma nitrite in fasted humans. Biochem Biophys Res Commun 1995;209:590-596.

194. Castillo L, Beaumier L, Ajami AM, Young VR. Whole body **nitric oxide** synthesis in healthy men determined from [^{15}N]arginine-to-[^{15}N]citrulline labeling. Proc Natl Acad Sci U S A 1996;93:11460-11465.

195. Veszelovsky E, Holford NHG, Thomsen LL, K**no**wles RG, Baguley BC. Plasma nitrate clearance in mice: modelling of the systemic production of nitrate following the induction of **nitric oxide** synthesis. Cancer Chemother Pharmacol 1995;36:155-159.

196. Steil AA, Garcia-Rodriguez MC, Alonso A, Crespo MS, Bosca L. Platelet-activating factor: the effector of protein-rich plasma extravasation and **nitric oxide** synthase induction in rat immune complex peritonitis. Br J Pharmacol 1995;114:895-901

197. Ribbons KA, Zhang X-J, Thompson JH, Greenberg SS, Moore WM, Kornmeier CM, et al. Potential role of **nitric oxide** in a model of chronic colitis in rhesus macaques. Gastroenterology 1995;108:705-711.

198. Rachmilewitz D, Karmeli F, Eliakim R, Stalnikowicz R, Ackerman Z, Amir G, Stamler JS. Enhanced gastric **nitric oxide** synthase activity in duodenal ulcer patients. Gut 1994;35:1394-1397.

199. Chamulitrat W, Jordan SJ, Mason RP. **Nitric oxide** production during endotoxic shock in carbon tetrachloride-treated rats. Mol Pharmacol 1994;46:391-397.

200. Reinke LA, Moore DR, Kotake Y. Hepatic **nitric oxide** formation: spin trapping detection in biliary efflux. Anal Biochem 1996;243:8-14.

201. Wallis G, Brackett D, Lerner M, Kotake Y, Bolli R, McCay PB. In vivo spin trapping of **nitric oxide** generated in the small intestine, liver, and kidney during the development of endotoxemia: a time-course study. Shock 1996;6:274-278.

202. Miranda K,M Espey. A rapid simple spectrophotometric method for simultaneous detection of nitrate and nitrite. Nitric oxide 2001,5:62-71

203. Moshage H, Kok B, Huizenga R, PeterJansan . Nitrite and Nitrate determinations in Plasma :A Critical Evaluation. CLIN.CHEM ; 1995: 892 – 896 .

204. Gillam M. Sherman B, Ignarro L. A spectrophotometric assay for nitrate using NADPH oxidation by Aspergillus nitrate reductase. Anal Biochem.; 1993 : 359 – 365.

205. Moshage H, Kok B, Huizenga JR, Jansen PLM. Nitrite and **nitrate** determ**in**ations **in** plasma: a critical evaluation. Cl**in** Chem 1995;41:892-896.

206. Bories PN, Bories C. **Nitrate** determ**in**ation **in** biological fluids by an enzymatic one-step assay with **nitrate** reductase. Cl**in** Chem 1995;41:904-907.

207. Yang F, Troncy E, Francoeur M, V**in**et B, V**in**ay P, Czaika G, Blaise G. Effect of reduc**in**g agents and temperatures on conversion of nitrite and **nitrate** to nitric oxide and detection of NO by chemilum**in**escence. Cl**in** Chem 1997;43:657-662.

208. Green LC, Wagner DA, Glogowski J, Skipper PL, Whishnok JS, Tannenbaum SR.Analysis of **nitrate**, nitrite and [^{15}N]**nitrate in** biological fluids. Anal Biochem 1982;126:131-138

209. Griess, P. Ber .Deutsch chem. .Ges. 1879,12,426.

210. Shinn M,B. Ind. Engng.Chem. Analyt.Edn. 1941, 13,33.

211. . Ignarro, L. J.; Buga, G. M.; Wood, K. S.; Byrns, R. E.; Chaudhuri, G. Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. Proc. Natl. Acad Sci. USA 84:9265-9269; 1987.

212. Palmer, R. M. J.; Ferrige, A. G.; Moncada, S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature 327:524-526; 1987.

213. Arnold, W. P.; Mittal, C. K.; Katsuki, S.; Murad, F. Nitric oxide activates guanylate cyclase and increases guanosine 3':5'-cyclic monophosphate levels in various tissue preparations. Proceedings of the National Academy of Sciences of the United States of America 74:3203-3207; 1977

214. . Katsuki, S.; Arnold, W.; Mittal, C.; Murad, F. Stimulation of guanylate cyclase by sodium nitroprusside, nitroglycerin and nitric oxide in various tissue preparations and comparison to the effects of sodium azide and hydroxylamine. Journal of cyclic nucleotide research 3:23-35; 1977.

215. Murad, F.; Mittal, C. K.; Arnold, W. P.; Katsuki, S.; Kimura, H. Guanylate cyclase: activation by azide, nitro compounds, nitric oxide, and hydroxyl radical and inhibition by hemoglobin and myoglobin. Advances in cyclic nucleotide research 9:145-158; 1978.

216. Murad, F.; Arnold, W. P.; Mittal, C. K.; Braughler, J. M. Properties and regulation of guanylate cyclase and some proposed functions for cyclic GMP. Advances in cyclic nucleotide research 11:175-204; 1979.

217. Furchgott, R. F.; Zawadzki, J. V. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetycholine. Nature 288:373-376; 1980.

218. . Murad, F. Cyclic guanosine monophosphate as a mediator of vasodilation. The Journal of clinical investigation 78:1-5; 1986.

219. . Liu, Q.; S.S., G. Binding sites of nitric oxide synthases. Methods Enzymol 268:311-324; 1996.

220. . Russwurm, M.; Behrends, S.; Harteneck, C.; Koesling, D. Functional properties of a naturally occurring isoform of soluble guanylyl cyclase. The Biochemical journal 335 (Pt 1):125-130; 1998.

221. Budworth, J.; Meillerais, S.; Charles, I.; Powell, K. Tissue distribution of the human soluble guanylate cyclases. Biochemical and biophysical research communications 263:696-701; 1999.

222. Mergia, E.; Russwurm, M.; Zoidl, G.; Koesling, D. Major occurrence of the new alpha2beta1 isoform of NO-sensitive guanylyl cyclase in brain. Cellular signalling 15:189-195; 2003.

223. Nimmegeers, S.; Sips, P.; Buys, E.; Brouckaert, P.; Van de Voorde, J. Functional role of the soluble guanylyl cyclase alpha(1) subunit in vascular smooth muscle relaxation. Cardiovascular research 76:149-159; 2007.

224. . Lucas, K. A.; Pitari, G. M.; Kazerounian, S.; Ruiz-Stewart, I.; Park, J.; Schulz, S.; Chepenik, K. P.; Waldman, S. A. Guanylyl cyclases and signaling by cyclic GMP. Pharmacol Rev 52:375-414; 2000.

225. . Biel, M.; Seeliger, M.; Pfeifer, A.; Kohler, K.; Gerstner, A.; Ludwig, A.; Jaissle, G.; Fauser, S.; Zrenner, E.; Hofmann, F. Selective loss of cone function in mice lacking the cyclic nucleotide-gated channel CNG3. Proceedings of the National Academy of Sciences of the United States of America 96:7553-7557; 1999.

226. Boolell, M.; Allen, M. J.; Ballard, S. A.; Gepi-Attee, S.; Muirhead, G. J.; Naylor, A. M.; Osterloh, I. H.; Gingell, C. Sildenafil: an orally active type 5 cyclic GMP-specific phosphodiesterase inhibitor for the treatment of penile erectile dysfunction. International journal of impotence research 8:47-52; 1996.

227. . Humbert, P.; Niroomand, F.; Fischer, G.; Mayer, B.; Koesling, D.; Hinsch, K. D.; Gausepohl, H.; Frank, R.; Schultz, G.; Bohme, E. Purification of soluble guanylyl cyclase from bovine lung by a new immunoaffinity chromatographic method. European journal of biochemistry / FEBS 190:273-278; 1990.

228. Lee, Y. C.; Martin, E.; Murad, F. Human recombinant soluble guanylyl cyclase: expression, purification, and regulation. Proceedings of the National Academy of Sciences of the United States of America 97:10763-10768; 2000.

229. . Friebe, A.; Mergia, E.; Dangel, O.; Lange, A.; Koesling, D. Fatal gastrointestinal obstruction and hypertension in mice lacking nitric oxide-sensitive guanylyl cyclase. Proceedings of the National Academy of Sciences of the United States of America 104:7699-7704; 2007.

230. . Mergia, E.; Friebe, A.; Dangel, O.; Russwurm, M.; Koesling, D. Spare guanylyl cyclase NO receptors ensure high NO sensitivity in the vascular system. The Journal of clinical investigation 116:1731-1737; 2006.

231. Pyriochou, A.; Papapetropoulos, A. Soluble guanylyl cyclase: more secrets revealed. Cellular signalling 17:407-413; 2005

232. . Krumenacker, J. S.; Hyder, S. M.; Murad, F. Estradiol rapidly inhibits soluble guanylyl cyclase expression in rat uterus. Proceedings of the National Academy of Sciences of the United States of America 98:717-722; 2001.

233. . Kloss, S.; Srivastava, R.; Mulsch, A. Down-regulation of soluble guanylyl cyclase expression by cyclic AMP is mediated by mRNA-stabilizing protein HuR. Molecular pharmacology 65:1440-1451; 2004.

234. . Papapetropoulos, A.; Marczin, N.; Mora, G.; Milici, A.; Murad, F.; Catravas, J. D. Regulation of vascular smooth muscle soluble guanylate cyclase activity, mRNA, and protein levels by cAMP-elevating agents. Hypertension 26:696-704; 1995.

235. Papapetropoulos, A.; Abou-Mohamed, G.; Marczin, N.; Murad, F.; Caldwell, R. W.; Catravas, J. D. Downregulation of nitrovasodilator-induced cyclic GMP accumulation in cells exposed to endotoxin or interleukin-1 beta. British journal of pharmacology 118:1359-1366; 1996.

236. . Papapetropoulos, A.; Go, C. Y.; Murad, F.; Catravas, J. D. Mechanisms of tolerance to sodium nitroprusside in rat cultured aortic smooth muscle cells. British journal of pharmacology 117:147-155; 1996.

237. . Kimura, H.; Murad, F. Evidence for two different forms of guanylate cyclase in rat heart. The Journal of biological chemistry 249:6910-6916; 1974.

238. Evgenov, O. V.; Pacher, P.; Schmidt, P. M.; Hasko, G.; Schmidt, H. H.; Stasch, J. P. NO-independent stimulators and activators of soluble guanylate cyclase: discovery and therapeutic potential. Nat Rev Drug Discov 5:755-768; 2006.

239. Bryan, N. S.; Calvert, J. W.; Elrod, J. W.; Gundewar, S.; Ji, S. Y.; Lefer, D. J. Dietary nitrite supplementation protects against myocardial ischemia-reperfusion injury. Proceedings of the National Academy of Sciences of the United States of America 104:19144-19149; 2007.

240. Bryan, N. S.; Fernandez, B. O.; Bauer, S. M.; Garcia-Saura, M. F.; Milsom, A. B.; Rassaf, T.; Maloney, R. E.; Bharti, A.; Rodriguez, J.; Feelisch, M. Nitrite is a signaling molecule and regulator of gene expression in mammalian tissues. Nat Chem Biol 1:290-297; 2005.

241. . Zweier, J. L.; Wang, P.; Samouilov, A.; Kuppusamy, P. Enzyme-independent formation of nitric oxide in biological tissues. Nat Med 1:804-809; 1995.

242. Lundberg, J. O.; Weitzberg, E.; Lundberg, J. M.; Alving, K. Intragastric nitric oxide production in humans: measurements in expelled air. Gut 35:1543-1546; 1994.

243. Kleinbongard, P.; Dejam, A.; Lauer, T.; Rassaf, T.; Schindler, A.; Picker, O.; Scheeren, T.; Godecke, A.; Schrader, J.; Schulz, R.; Heusch, G.; Schaub, G. A.; Bryan, N. S.; Feelisch, M.; Kelm, M. Plasma nitrite reflects constitutive nitric oxide synthase activity in mammals. Free Radic Biol Med 35:790-796; 2003.

244. . Gladwin, M. T.; Schechter, A. N.; Kim-Shapiro, D. B.; Patel, R. P.; Hogg, N.; Shiva, S.; Cannon, R. O., 3rd; Kelm, M.; Wink, D. A.; Espey, M. G.; Oldfield, E. H.; Pluta, R. M.; Freeman, B. A.; Lancaster, J. R., Jr.; Feelisch, M.; Lundberg, J. O. The emerging biology of the nitrite anion. Nat Chem Biol 1:308-314; 2005.

245. Bryan, N. S. Nitrite in nitric oxide biology: Cause or consequence? A systems-based review. Free Radic Biol Med 41:691-701; 2006.

246. . Tannenbaum, S. R.; Sinskey, A. J.; Weisman, M.; Bishop, W. Nitrite in human saliva. Its possible relationship to nitrosamine formation. J Natl Cancer Inst 53:79-84; 1974.

247. van Maanen, J. M.; van Geel, A. A.; Kleinjans, J. C. Modulation of nitrate-nitrite conversion in the oral cavity. Cancer Detect Prev 20:590-596; 1996

248. . Bryan, N. S.; Rassaf, T.; Maloney, R. E.; Rodriguez, C. M.; Saijo, F.; Rodriguez, J. R.; Feelisch, M. Cellular Targets and Mechanisms of Nitros(yl)ation: An Insight into Their Nature and Kinetics in vivo. Proc. Natl. Acad Sci. USA 101:4308-4313; 2004.

249. . Zweier, J. L.; Wang, P.; Samouilov, A.; Kuppusamy, P. Enzyme-independent formation of nitric oxide in biological tissues. Nat Med 1:804-809; 1995.

250. . Lundberg, J. O.; Weitzberg, E. NO generation from nitrite and its role in vascular control. Arterioscler Thromb Vasc Biol 25:915-922; 2005.

251. Webb, A.; Bond, R.; McLean, P.; Uppal, R.; Benjamin, N.; Ahluwalia, A. Reduction of nitrite to nitric oxide during ischemia protects against myocardial ischemia-reperfusion damage. Proc Natl Acad Sci U S A 101:13683-13688; 2004.

252. Duranski, M. R.; Greer, J. J.; Dejam, A.; Jaganmohan, S.; Hogg, N.; Langston, W.; Patel, R. P.; Yet, S. F.; Wang, X.; Kevil, C. G.; Gladwin, M. T.; Lefer, D. J. Cytoprotective effects of nitrite during in vivo ischemia-reperfusion of the heart and liver. J Clin Invest 115:1232-1240; 2005.

253. Pluta, R. M.; Dejam, A.; Grimes, G.; Gladwin, M. T.; Oldfield, E. H. Nitrite infusions to prevent delayed cerebral vasospasm in a primate model of subarachnoid hemorrhage. Jama 293:1477-1484; 2005.

254. Hunter, C. J.; Dejam, A.; Blood, A. B.; Shields, H.; Kim-Shapiro, D. B.; Machado, R.; Tarekegn, S.; Mulla, N.; Hopper, A. O.; Schechter, A. N.; Power, G. G.; Gladwin, M. T. Inhaled nebulized nitrite is a hypoxia-sensitive NO-dependent selective pulmonary vasodilator. Nat Med 10:1122-1127; 2004.

255. Hardwick, J. B.; Tucker, A. T.; Wilks, M.; Johnston, A.; Benjamin, N. A novel method for the delivery of nitric oxide therapy to the skin of human subjects using a semi-permeable membrane. Clin Sci (Lond) 100:395-400; 2001.

256. Tsuchiya, K.; Kanematsu, Y.; Yoshizumi, M.; Ohnishi, H.; Kirima, K.; Izawa, Y.; Shikishima, M.; Ishida, T.; Kondo, S.; Kagami, S.; Takiguchi, Y.; Tamaki, T. Nitrite is an alternative source of NO in vivo. Am J Physiol Heart Circ Physiol 288:H2163-2170; 2005.

257. Kleinbongard, P.; Dejam, A.; Lauer, T.; Jax, T.; Kerber, S.; Gharini, P.; Balzer, J.; Zotz, R. B.; Scharf, R. E.; Willers, R.; Schechter, A. N.; Feelisch, M.; Kelm, M. Plasma nitrite concentrations reflect the degree of endothelial dysfunction in humans. Free Radic Biol Med 40:295-302; 2006.

258. . Pennington, J. A. T. Dietary exposure models for nitrates and nitrites. Food Control 9:385-395; 1998.

259. Foster, M. W.; McMahon, T. J.; Stamler, J. S. S-nitrosylation in health and disease. Trends in molecular medicine 9:160-168; 2003.

260. Massy, Z. A.; Fumeron, C.; Borderie, D.; Tuppin, P.; Nguyen-Khoa, T.; Benoit, M. O.; Jacquot, C.; Buisson, C.; Drueke, T. B.; Ekindjian, O. G.; Lacour, B.; Iliou, M. C. Increased pasma S-nitrosothiol concentrations predict cardiovascular outcomes among patients with end-stage renal disease: a prospective study. J Am Soc Nephrol 15:470-476; 2004.

261. . Heiss, C.; Lauer, T.; Dejam, A.; Kleinbongard, P.; Hamada, S.; Rassaf, T.; Matern, S.; Feelisch, M.; Kelm, M. Plasma nitroso compounds are decreased in

patients with endothelial dysfunction. Journal of the American College of Cardiology 47:573-579; 2006.

262. Hess, D. T.; Matsumoto, A.; Kim, S. O.; Marshall, H. E.; Stamler, J. S. Protein S-nitrosylation: purview and parameters. Nature reviews 6:150-166; 2005.

263. Hara, M. R.; Agrawal, N.; Kim, S. F.; Cascio, M. B.; Fujimuro, M.; Ozeki, Y.; Takahashi, M.; Cheah, J. H.; Tankou, S. K.; Hester, L. D.; Ferris, C. D.; Hayward, S. D.; Snyder, S. H.; Sawa, A. S-nitrosylated GAPDH initiates apoptotic cell death by nuclear translocation following Siah1 binding. Nature cell biology 7:665-674; 2005.

264. Whalen, E. J.; Foster, M. W.; Matsumoto, A.; Ozawa, K.; Violin, J. D.; Que, L. G.; Nelson, C. D.; Benhar, M.; Keys, J. R.; Rockman, H. A.; Koch, W. J.; Daaka, Y.; Lefkowitz, R. J.; Stamler, J. S. Regulation of beta-Adrenergic Receptor Signaling by S-Nitrosylation of G-Protein-Coupled Receptor Kinase 2. Cell 129:511-522; 2007.

265. Kelleher, Z. T.; Matsumoto, A.; Stamler, J. S.; Marshall, H. E. NOS2 regulation of NF-kappaB by S-nitrosylation of p65. J Biol Chem 282:30667-30672; 2007.

266. Liu, L.; Hausladen, A.; Zeng, M.; Que, L.; Heitman, J.; Stamler, J. S. A metabolic enzyme for S-nitrosothiol conserved from bacteria to humans. Nature 410:490-494; 2001.

267. . Pacher, P.; Beckman, J. S.; Liaudet, L. Nitric oxide and peroxynitrite in health and disease. Physiol Rev 87:315-424; 2007.

268. Turko, I. V.; Murad, F. Protein nitration in cardiovascular diseases. Pharmacol Rev 54:619-634; 2002.

269. . Souza, J. M.; Daikhin, E.; Yudkoff, M.; Raman, C. S.; Ischiropoulos, H. Factors determining the selectivity of protein tyrosine nitration. Arch Biochem Biophys 371:169-178; 1999.

270. Wu, W.; Chen, Y.; Hazen, S. L. Eosinophil peroxidase nitrates protein tyrosyl residues. Implications for oxidative damage by nitrating intermediates in eosinophilic inflammatory disorders. J Biol Chem 274:25933-25944; 1999.

271. Sampson, J. B.; Ye, Y.; Rosen, H.; Beckman, J. S. Myeloperoxidase and horseradish peroxidase catalyze tyrosine nitration in proteins from nitrite and hydrogen peroxide. Arch Biochem Biophys 356:207-213; 1998.

272. Eiserich, J. P.; Hristova, M.; Cross, C. E.; Jones, A. D.; Freeman, B. A.; Halliwell, B.; van der Vliet, A. Formation of nitric oxide-derived inflammatory oxidants by myeloperoxidase in neutrophils. Nature 391:393-397; 1998.

273. Carr, A. C.; Frei, B. The nitric oxide congener nitrite inhibits myeloperoxidase/H2O2/ Cl- -mediated modification of low density lipoprotein. J Biol Chem 276:1822-1828; 2001.

274. Bian, K.; Gao, Z.; Weisbrodt, N.; Murad, F. The nature of heme/iron-induced protein tyrosine nitration. Proc Natl Acad Sci U S A 100:5712-5717; 2003.

275. .Moncada S, Higgs A. The L-arginine-nitric oxide pathway. N Engl J Med. 1993;329:2002-2012.

276. Huang PL, Huang ZH, Mashimo H, et al. Hypertension in mice lacking the gene for endothelial nitric oxide synthase. Nature. 1995;377:239-242.

277. . Benjafield AV, Morris BJ. Association analysis of endothelial nitric oxide synthase gene polymorphisms is essential in hypertension. Am J Hypertens. 2000;13:994-998.

278. . Ohashi Y, Kawashima S, Hirata K, et al. Hypotension and reduced nitric oxide-elicited vasorelaxation in transgenic mice overexpressing endothelial nitric oxide synthase. J Clin Invest. 1998;102:2061-2071.

279. . Lin KF, Chao L, Chao J. Prolonged reduction of high blood pressure with human nitric oxide synthase gene delivery. Hypertension. 1997;30:307-313.

280. Balon TW, Nadler JL. Evidence that nitric oxide increases glucose transport in skeletal muscle. J Appl Physiol. 1997;82:359-363.

281. . Laufs U, Lafata V, Plutzky J, Liao JK. Upregulation of endothelial nitric oxide synthase by HMG CoA reductase inhibitors. Circulation. 1998;97:1129-1135.

282. . Weiner CP, Lizasoain I, Baylis SA, Knowles RG, Charles IG, Moncada S. Induction of calcium-dependent nitric oxide synthases by sex hormones. Proc Natl Acad Sci U S A. 1994;91:5212-5216.

283. Cheitlin MD, Hutter AM Jr, Brindis RG, et al for the Technology and Practice Executive Committee. Use of sildenafil (Viagra) in patients with cardiovascular disease. Circulation. 1999;99:168-177.

284. Calver A, Collier J, Leone A, Moncada S, Vallance P. Effect of local intra-arterial asymmetric dimethylarginine (ADMA) on the forearm arteriolar bed of healthy volunteers. J Hum Hypertens. 1993;7:193-194.

285. Vallance P, Leone A, Calver A, Collier J, Moncada S. Accumulation of an endogenous inhibitor of nitric oxide synthesis in chronic renal failure. Lancet. 1992;339:572-575.

286. Chen PY, Sanders PW. L-arginine abrogates salt-sensitive hypertension in Dahl/Rapp rats. J Clin Invest. 1991;88:1559-1567.

287. Wever R, Boer P, Hijmering M, et al. Nitric oxide production is reduced in patients with chronic renal failure. Arterioscler Thromb Vasc Biol. 1999;19:1168-1172.

288. Griendling KK, Sorescu D, Ushio-Fukai M. NAD(P)H oxidase. Circ Res. 2000;86:494-501.

289. Laursen JB, Rajagopalan S, Galis Z, Tarpey M, Freeman BA, Harrison DG. Role of superoxide in angiotensin II-induced but not catecholamine-induced hypertension. Circulation. 1997;95:588-593.

290. Schnackenberg CG, Welch WJ, Wilcox CS. Normalization of blood pressure and renal vascular resistance in SHR with a membrane-permeable superoxide dismutase mimetic. Hypertension. 1998;32:59-64.

291. Duffy SJ, Gokce N, Holbrook M, et al. Treatment of hypertension with ascorbic acid. Lancet. 1999;354:2048-2049.

292. Ness AR, Chee D, Elliott P. Vitamin C and blood pressure —an overview. J Hum Hypertens. 1997;11:343-350.

293. Fukai T, Siegfried MR, Ushio-Fukai M, Cheng Y, Kojda G, Harrison DG. Regulation of the vascular extracellular superoxide dismutase by nitric oxide and exercise training. J Clin Invest. 2000;105:1631-1639.

294. Gail D. Thomas, PhD; Weiguo Zhang, MD, PhD; Ronald G. Victor, MD . Nitric Oxide Deficiency as a Cause of Clinical HypertensionPromising New Drug Targets for Refractory Hypertension. JAMA. 2001;285:2055-2057

295. Surdacki, Andrzej; Nowicki, Michał. Reduced Urinary Excretion of Nitric Oxide Metabolites and Increased Plasma Levels of Asymmetric

Dimethylarginine in Men with Essential Hypertension Journal of Cardiovascular Pharmacology;1999: 652-658.

296. Panza JA, Casino PR, Badar DM , Quyyumi AA. . Effect of increased availability of endothelium-derived nitric oxide precursor on endothelium-dependent vascular relaxation in normal subjects and in patients with essential hypertension. Circulation; Vol 87: 1475-1481.

297. Thomsen, L. L., Scott, J. M., Topley, P., Knowles, R. G., Keerie, A. J. and Frend, A. J. (1997) Selective inhibition of inducible nitric oxide synthase inhibits tumor growth *in vivo*: studies with 1400W, a novel inhibitor. Cancer Res. **57**, 3300–3304

298. Wray, G. M., Millar, C. G., Hinds, C. J. and Thiemermann, C. (1998) Selective inhibition of the activity of inducible nitric oxide synthase prevents the circulatory failure, but not the organ injury/dysfunction, caused by endotoxin. Shock **9**, 329–335

299. . Parmentier, S., Bohme, G. A., Lerouet, D., Damour, D., Stutzmann, J. M., Margaill, I. and Plotkine, M. (1999) Selective inhibition of inducible nitric oxide synthase prevents ischaemic brain injury. Br. J. Pharmacol. **127**, 546–552

300. Koarai, A., Ichinose, M., Sugiura, H., Yamagata, S., Hattori, T. and Shirato, K. (2000) Allergic airway hyperresponsiveness and eosinophil infiltration is reduced by a selective iNOS inhibitor, 1400W, in mice. Pulmon. Pharmacol. Ther. **13**, 267–275

301. Kankuri, E., Vaali, K., Knowles, R., Lähde, M., Korpela, R., Vapaatalo, H. and Moilanen, E. (2001) Suppression of acute experimental colitis by an iNOS-selective inhibitor *N*-3-(aminomethyl)benzyl]acetamidine (1400W). J. Pharmacol. Exp. Ther., in the press

302. Young, R. J., Beams, R. M., Carter, K., Clark, H. A., Coe, D. M., Chambers, C. L., Davies, P. I., Dawson, J., Drysdale, M. J., Franzman, K. W. (2000) Inhibition of inducible nitric oxide synthase by acetamidine derivatives of hetero-substituted lysine and homolysine. Bioorg. Med. Chem. Lett. **10**, 597–600

303. Dawson, J. and Knowles, R. G. (1998) A microtiter-plate assay of human NOS isoforms. Methods Mol. Biol. **100**, 237–242

304. Friedewald WT , Levi RI , Fredrickson DS . Estimation of low density lipoprotein cholesterol levels in plasma without use of the preparative ultracentrifuge. Clin Chem. 1972 ;18:499-502.

305. Lander ES , Schor NJ . Genetic dissection of complex traits . Science ..1994;265:2037-2048.

306. Gonzálvez JA, Benayas CG, and Arenas J. Semiautomated Measurement of Nitrate in Biological Fluids .Clin Chem 1998; 44:679-681.

307. Sorte K and Basak A . Development of a modified copper-cadmium reduction method for rapid assay of total nitric oxide . Anal. Methods. 2010; 2: 944-947

308. Hingorani AD, Liang CF, Fatibene J, Lyon A, Monteith S, Parsons A, *et al* . A common Vairant of endothelial nitric oxide synthase (Glu 298-Asp) is a major risk factor for coronary artery disease in UK. Circulation 1999;100:1515-20.

309. Colombo MG, Paradossi U, Andreassi MG, Botto N, Manfredi S, Masetti S, *et al* . Endothelial nitric oxide synthase gene polymorphisms and risk of coronary artery disease. Clin Chem 2003;49:389-95.

310. Hyndman ME, Parsons HG, Verma S, Bridge PJ, Edworthy S, Jones C, *et al* . The T-786 C mutatation in endothelial nitric oxide synthase is associatd with hypertension. Hypertension 2002;39:919-22.

311. Nakayama M, Yasue H, Yashimura M, Shimasaki Y, Kugiyama K, Ogawa H, *et al* . T786C mutation in the 5´-flanking region of the endothelial nitric oxide synthase Gene is associated with coronary spasm. Circulation 1999;99:2864-70.

312. Wang XL, Sim AS, Badenhop RF, McCredie RM, Wilcken DE. A smoking dependant risk of coronary artery disease associated with a polymorphism of the endothelial nitric oxide synthase gene. Nat Med 1996;2:41-5.

313. Wang Y, Kikuchi K, Suzuki H, Nagase S, Koyama A. Endothelial Nitric Oxide Synthase gene Polymorphism in intron 4 affects the progression of renal failure in Non - diabetic renal diseases. Nephrol Dial Transplant 1999;14:2898-902.

314. Buraczнska M, Ksiazek P, Zaluska W, Nowicka T, Ksiazek A. Endothelial nitric oxide synthase gene intron 4 polymorphism in patients with end stage renal disease. Nephrol Dial Transplant 2004;19:2302-6.

315. Uwabo J, Soma M, Nakayama T, Kanmatsuse K. Association of a Variable Number of tandem repeats in Endotheial Constitutive Nitric Oxide Synthase gene with essential hypertension. Am J Hypertns 1998;11:125-8.

316. Hou L, Osei-Hyiaman D,Yu H, Ren Z, Zhang Z, Wang B, Harada S. Association of a 27 bp repeat polymorphism in *eNOS* gene with ischemic stroke in Chinese Population. Neurology 2001;56:4.

317. Yen WenWu, Lee C, Hsu S, Lee Y. Association of endothelial nitric oxide synthase polymorphism and the risk of premature coronary artry disease in taiwan. J Inter Med Taiwan 2003;14:1-10.

ABBREVIATIONS

ABBREVIATIONS

CHD	**-**	**coronary heart disease**
VLDL	**-**	**very low density lipoprotein**
LDL	**-**	**low density lipoprotein**
HDL	**-**	**high density lipoprotein**
EDRF	**-**	**endothelium derived relaxing factor**
MLCK	**-**	**myosin light chain kinase**
sGC	**-**	**soluble guanylyl cyclase**
PDE	**-**	**phosphodiesterase**
NO.	**-**	**nitric oxide**
cGMP	**-**	**cyclic guanosine mono-phosphate**
GTP	**-**	**guanosine tri-phosphate**
cAMP	**-**	**cyclic adenosine mono-phosphate**
eNOS	**-**	**endothelial nitric oxide synthase**
iNOS	**-**	**inducible nitric oxide synthase**
nNOS	**-**	**neuronal nitric oxide synthase**

RAAS	**-**	**renin angiotensin aldesterone system**
ACE	**-**	**angiotensin converting enzyme**
ENAC	**-**	**epithelial sodium channels**
OD	**-**	**optical density**
L-NAME	**-**	**L-NG Nitroarginine methyl ester**
RSNO	**-**	**S-nitrosothiols**
GSNOR	**-**	**S-nitroso glutathione reductase**
MPO	**-**	**myeloperoxidase**
ADMA	**-**	**asymmetric dimethyl arginine**
BH4	**-**	**tetra-hydro-biopterin**
FAD	**-**	**flavin adenine di-nucleotide**
FMN	**-**	**flavin mono-nucleotide**
CaM	**-**	**calmodulin**
hsp	**-**	**heat shock protein**
IP3	**-**	**inositol 1,4,5 triphosphate**
PRD	**-**	**positive regulatory domain**

TGF	**-**	**transforming growth factor**
BAEC	**-**	**bovine aortic endothelial cells**
HUVEC	**-**	**human unbilical vein endothelial cells**
TNF	**-**	**tumor necrosing factor**
Ox-LDL	**-**	**oxidized - low density lipoprotein**
VEGF	**-**	**vascular endothelial growth factor**
TSA	**-**	**trichostatin A**
EPR	**-**	**electron paramagnetic resonance**
GC-MS	**-**	**gas chromatography- mass spectrometry**
HPLC	**-**	**high performance liquid chromatography**
NOx	**-**	**nitrate+ nitrite**
NR	**-**	**nitrate reductase**
G6PD	**-**	**glucose-6-phosphate dehydrogenase**
NED	**-**	**N-naphthyl-ethylene diamine**

Table 1

Master chart for cases

7	Age	Sex	Smoking	Alcoholism	Wt. (Kg)	Height (m)	Ht. (cm)	BMI	Blood sugar fasting (mg/dl)	serum urea (mg/dl)	serum creatinine (mg/dl)	CHOL (mg/dl)	TGL (mg/dl)	HDL (mg/dl)	LDL (mg/dl)	SERUM TOTAL NITRITES(µmol/L)	eNOS intron4 genotype
1	75	M	No	No	60	1.6	160	23.44	94	40	0.68	143	173	30	78.4	17.2	b/b
2	54	M	No	No	80	1.56	156	32.87	102	26	0.78	218	355	33	114	12.8	b/b
3	60	M	No	No	65	1.6	160	25.39	97	36	0.59	212	285	31	124	13.1	b/b
4	60	M	No	No	65	1.53	153	27.77	106	38	0.68	104	98	29	55.4	9.8	b/b
5	75	M	No	No	72	1.61	161	27.78	102	25	0.87	164	266	35	75.8	21.1	b/b
6	54	M	No	No	56	1.66	166	20.32	84	29	0.96	159	110	52	85	22.1	b/b
7	61	M	No	No	60	1.66	166	21.77	96	36	1.1	207	155	27	149	8.9	a/b
8	44	M	No	No	74	1.59	159	29.27	92	38	1.06	221	154	36	154.2	14	b/b
9	53	M	No	No	68	1.64	164	25.28	102	37	0.76	145	130	34	85	6.7	a/a
10	34	M	Yes	Yes	65	1.55	155	27.06	101	36	0.59	208	260	54	102	9.8	a/b
11	60	M	No	No	75	1.65	165	27.55	104	26	0.76	136	189	37	61.2	11.8	b/b
12	38	M	Yes	Yes	59	1.6	160	23.05	100	29	0.89	238	147	57	151.6	23.7	a/b
13	57	M	No	No	87	1.7	170	30.10	98	34	0.96	212	188	32	142.4	10.2	b/b
14	45	F	No	No	86	1.57	157	34.89	93	32	0.95	153	108	29	102.4	13	b/b
15	52	F	No	No	74	1.48	148	33.78	86	31	0.86	188	183	30	121.4	6	a/b
16	60	M	No	No	69	1.68	168	24.45	103	25	0.79	195	250	25	120	17.8	b/b
17	55	M	No	No	61	1.63	163	22.96	104	36	0.69	193	275	28.6	109.4	15.4	b/b
18	61	M	No	No	63	1.54	154	26.56	95	37	0.87	185	132	32.6	126	9.8	a/b
19	40	M	No	No	66	1.67	167	23.67	84	24	1.04	149	183	23.8	88.6	9.7	a/a
20	48	M	No	No	59	1.68	168	20.90	89	25	0.9	198	98	31	147.4	21.3	b/b
21	54	M	No	No	77	1.7	170	26.64	103	37	0.86	134	130	53.4	54.6	10.9	a/b
22	50	M	No	No	65	1.65	165	23.88	95	25	0.76	130	109	52	56.2	17.9	b/b
23	67	M	No	No	57	1.6	160	22.27	108	31	0.59	116	85	43	56	16.9	b/b
24	48	M	Yes	No	55	1.65	165	20.20	88	26	0.96	194	137	39.6	127	19.5	b/b
25	43	M	Yes	Yes	60	1.65	165	22.04	97	29	0.58	127	176	36	55.8	21.6	b/b
26	60	M	No	No	96	1.6	160	37.50	89	34	0.96	143	138	33	82.4	10.3	a/b
27	35	F	No	No	64	1.55	155	26.64	105	31	0.95	173	102	42.2	110.4	16.9	b/b
28	55	M	No	No	54	1.53	153	23.07	93	25	0.87	146	271	34.3	57.5	13.8	b/b
29	40	M	No	No	67	1.71	171	22.91	98	37	1.03	212	194	38	135.2	16.4	b/b
30	47	M	Yes	No	73	1.63	163	27.48	105	40	1	206	153	41	134.4	11.1	a/b
31	56	M	No	No	75	1.61	161	28.93	95	32	0.97	147	113	33.5	90.9	15.7	b/b
32	63	M	No	No	67	1.72	172	22.65	101	31	0.9	114	201	45	28.8	18.9	b/b
33	52	M	No	No	75	1.71	171	25.65	86	35	0.8	123	197	52	31.6	11.7	a/b
34	59	M	No	No	62	1.68	168	21.97	94	37	0.84	149	187	28.1	83.5	21.9	b/b
35	52	M	No	No	64	1.65	165	23.51	102	26	0.71	120	134	34	59.2	10.8	a/b
36	55	M	No	No	60	1.63	163	22.58	84	27	0.73	149	123	36.4	88	9.8	a/b
37	70	M	No	No	53	1.52	152	22.94	96	36	0.69	156	130	38.2	91.8	5.8	a/a
38	53	M	No	No	56	1.63	163	21.08	92	27	0.93	138	156	26.4	80.4	11.3	a/b
39	53	M	Yes	Yes	55	1.62	162	20.96	102	28	0.82	143	151	33.6	79.2	17.5	b/b
40	28	M	No	No	52	1.56	156	21.37	98	37	0.74	138	162	45.1	60.5	11.4	a/b
41	63	M	No	No	54	1.66	166	19.60	92	25	0.69	162	92	38	105.6	17.9	b/b

42	58	M	No	No	64	1.55	155	26.64	100	33	0.95	189	171	24	130.8	12.1	a/b
43	25	M	Yes	No	55	1.58	158	22.03	98	26	0.94	166	154	39	96.2	9.7	a/a
44	51	M	No	No	63	1.76	176	20.34	93	28	0.8	145	167	32	79.6	9.9	a/b
45	48	M	Yes	Yes	58	1.67	167	20.80	96	22	0.87	175	166	28	113.8	16.9	b/b
46	45	M	Yes	Yes	72	1.76	176	23.24	92	32	0.6	126	152	38.3	57.3	19.4	b/b
47	58	M	No	No	49	1.48	148	22.37	102	28	0.98	125	136	43	54.8	7.3	a/a
48	35	M	No	No	72	1.64	164	26.77	108	31	0.98	181	138	37.3	116.1	20.1	b/b
49	50	M	No	No	51	1.55	155	21.23	104	28	0.7	175	192	41	95.6	18.4	b/b
50	51	M	No	No	57	1.56	156	23.42	100	29	0.86	151	148	45	76.4	7.5	a/b
51	54	F	No	No	75	1.51	151	32.89	98	37	0.92	185	143	37	119.4	16.2	b/b
52	60	F	No	No	75	1.53	153	32.04	102	25	0.85	146	153	36	79.4	17.5	b/b
53	50	M	Yes	No	65	1.7	170	22.49	83	31	0.84	148	182	38	73.6	9.3	a/b
54	52	M	Yes	No	80	1.7	170	27.68	85	39	0.93	168	174	28	105.2	16.9	b/b
55	29	M	Yes	Yes	65	1.69	169	22.76	93	35	1.05	156	182	35	84.6	24.9	b/b
56	50	M	No	No	68	1.78	178	21.46	97	27	0.78	156	173	43	78.4	19.9	b/b
57	47	M	No	No	70	1.62	162	26.67	89	22	1.09	165	168	51	80.4	11.7	a/b
58	50	M	No	No	90	1.71	171	30.78	88	39	0.76	174	142	34	111.6	17.6	b/b
59	34	M	Yes	Yes	76	1.76	176	24.54	89	24	0.98	125	163	55	37.4	17.6	b/b
60	50	M	No	No	62	1.67	167	22.23	95	26	0.9	141	149	38	73.2	14.9	b/b
61	33	M	Yes	Yes	68	1.63	163	25.59	100	27	0.89	117	199	35.4	41.8	11.4	a/b
62	48	M	Yes	No	55	1.65	165	20.20	88	25	0.84	194	137	39.6	127	21.2	b/b
63	43	M	Yes	Yes	60	1.65	165	22.04	97	28	0.82	127	176	36	55.8	17.3	b/b
64	60	M	No	No	96	1.6	160	37.50	104	38	0.95	143	138	33	82.4	9.5	a/b
65	35	F	No	No	64	1.55	155	26.64	98	29	0.9	173	102	42.2	110.4	14.8	b/b
66	55	M	No	No	54	1.53	153	23.07	93	24	0.85	146	271	34.3	57.5	10.5	a/b
67	40	M	No	No	67	1.71	171	22.91	98	36	0.7	212	194	38	135.2	15.9	b/b
68	47	M	Yes	No	73	1.63	163	27.48	105	39	0.75	206	153	41	134.4	8.4	a/b
69	56	M	No	No	75	1.61	161	28.93	95	25	0.96	147	113	33.5	90.9	16.9	b/b
70	63	M	No	No	67	1.72	172	22.65	101	31	0.94	114	201	45	28.8	18.6	b/b
71	52	M	No	No	75	1.71	171	25.65	86	36	0.92	123	197	52	31.6	19.1	b/b
72	59	M	No	No	62	1.68	168	21.97	94	23	0.69	149	187	28.1	83.5	16.9	b/b
73	52	M	No	No	64	1.65	165	23.51	102	32	0.74	120	134	34	59.2	11.4	b/b
74	55	M	No	No	60	1.63	163	22.58	84	36	0.83	149	123	36.4	88	8.8	a/b
75	70	M	No	No	53	1.52	152	22.94	96	28	0.91	156	130	38.2	91.8	9.6	a/b
76	46	M	No	No	72	1.63	163	27.10	98	36	1.03	188	151	38	182	15.8	b/b
77	56	F	No	No	62	1.68	168	21.97	102	35	1.1	141	168	38	110	21.9	a/b
78	65	M	Yes	Yes	69	1.71	171	23.60	87	36	0.99	191	201	35	160	10.8	a/b
79	46	F	No	No	70	1.61	161	27.01	98	26	0.79	188	151	38	182	19.8	b/b
80	59	M	No	Yes	73	1.72	172	24.68	103	28	1.03	211	186	38	157	5.8	a/b
81	35	F	No	No	64	1.55	155	26.64	106	39	0.96	173	102	42.2	110.4	11.3	b/b
82	29	M	Yes	Yes	65	1.69	169	22.76	93	35	0.85	156	182	35	84.6	17.5	b/b
83	50	M	No	No	68	1.78	178	21.46	107	36	0.77	156	173	43	78.4	11.4	a/b
84	47	M	No	No	70	1.62	162	26.67	105	38	0.8	165	168	51	80.4	17.9	b/b
85	50	M	No	No	90	1.71	171	30.78	88	26	0.94	174	142	34	111.6	12.1	b/b
86	34	M	Yes	Yes	76	1.76	176	24.54	89	27	0.84	125	163	55	37.4	17.5	b/b

87	50	M	No	No	62	1.67	167	22.23	95	29	0.86	141	149	38	73.2	9.9	a/b
88	33	M	Yes	Yes	68	1.63	163	25.59	98	32	0.94	117	199	35.4	41.8	16.9	b/b
89	48	M	Yes	No	55	1.65	165	20.20	88	27	0.98	194	137	39.6	127	19.4	b/b
90	43	M	Yes	Yes	60	1.65	165	22.04	97	31	0.7	127	176	36	55.8	17.6	b/b
91	60	M	No	No	96	1.6	160	37.50	94	28	0.86	143	138	33	82.4	10.3	a/b
92	35	F	No	No	64	1.55	155	26.64	104	28	0.8	173	102	42.2	110.4	18.4	b/b
93	54	M	No	No	80	1.56	156	32.87	102	25	0.86	218	355	33	114	16.4	b/b
94	60	M	No	No	65	1.6	160	25.39	97	31	0.87	212	285	31	124	16.2	b/b
95	60	M	No	No	65	1.53	153	27.77	97	27	0.96	104	98	29	55.4	17.5	b/b
96	75	M	No	No	72	1.61	161	27.78	102	24	0.8	164	266	35	75.8	11.9	b/b
97	54	M	No	No	56	1.66	166	20.32	84	27	1.12	159	110	52	85	16.9	b/b
98	61	M	No	No	60	1.66	166	21.77	96	20	1.14	207	155	27	149	9.5	a/b
99	44	M	No	No	74	1.59	159	29.27	92	24	0.95	221	154	36	154.2	19.9	b/b
100	53	M	No	No	68	1.64	164	25.28	102	31	0.93	145	130	34	85	21.6	b/b
101	34	M	Yes	Yes	65	1.55	155	27.06	108	34	0.78	208	260	54	102	13.1	b/b
102	63	M	No	No	67	1.72	172	22.65	94	25	0.59	114	201	45	28.8	9.8	b/b
103	52	M	No	No	75	1.71	171	25.65	86	27	0.68	123	197	52	31.6	9.7	a/b
104	59	M	No	No	62	1.68	168	21.97	94	28	0.87	149	187	28.1	83.5	22.1	b/b
105	52	M	No	No	64	1.65	165	23.51	102	25	0.96	120	134	34	59.2	18.7	b/b
106	55	M	No	No	60	1.63	163	22.58	84	36	1.1	149	123	36.4	88	14	b/b
107	70	M	No	No	53	1.52	152	22.94	96	34	0.71	156	130	38.2	91.8	6.7	a/b
108	46	M	No	No	72	1.63	163	27.10	98	32	0.73	188	151	38	182	16.3	b/b
109	56	F	No	No	62	1.68	168	21.97	102	31	0.69	141	168	38	110	11.8	a/b
110	65	M	Yes	Yes	69	1.71	171	23.60	94	21	0.93	191	201	35	160	23.7	b/b
111	46	F	No	No	70	1.61	161	27.01	87	25	0.82	188	151	38	182	10.2	b/b
112	59	M	No	Yes	73	1.72	172	24.68	83	27	0.74	211	186	38	157	13	b/b
113	35	F	No	No	64	1.55	155	26.64	103	31	0.69	173	102	42.2	110.4	16.1	b/b
114	29	M	Yes	Yes	65	1.69	169	22.76	93	28	0.95	156	182	35	84.6	9.4	a/b
115	50	M	No	No	68	1.78	178	21.46	95	28	0.94	156	173	43	78.4	15.4	b/b
116	47	M	No	No	70	1.62	162	26.67	105	25	0.8	165	168	51	80.4	15.9	b/b
117	50	M	No	No	90	1.71	171	30.78	88	31	0.79	174	142	34	111.6	17.8	b/b
118	34	M	Yes	Yes	76	1.76	176	24.54	89	27	0.7	125	163	55	37.4	5.7	a/a
119	50	M	No	No	62	1.67	167	22.23	95	24	0.75	141	149	38	73.2	13.3	b/b
120	33	M	Yes	Yes	68	1.63	163	25.59	98	27	0.79	117	199	35.4	41.8	8.3	a/b
121	48	M	Yes	No	55	1.65	165	20.20	88	20	0.84	194	137	39.6	127	16.9	b/b
122	38	M	Yes	Yes	59	1.6	160	23.05	100	24	0.95	238	147	57	151.6	19.5	b/b
123	57	M	No	No	87	1.7	170	30.10	98	26	0.58	212	188	32	142.4	21.6	b/b
123	45	F	No	No	86	1.57	157	34.89	93	21	0.63	153	108	29	102.4	12.7	b/b
124	52	F	No	No	74	1.48	148	33.78	86	22	0.94	188	183	30	121.4	16.9	b/b
125	60	M	No	No	69	1.68	168	24.45	99	34	0.68	195	250	25	120	11	a/b
126	55	M	No	No	61	1.63	163	22.96	104	31	1.04	193	275	28.6	109.4	16.4	b/b
127	61	M	No	No	63	1.54	154	26.56	95	37	0.84	185	132	32.6	126	17.4	b/b
128	40	M	No	No	66	1.67	167	23.67	84	26	1.12	149	183	23.8	88.6	15.7	b/b
129	48	M	No	No	59	1.68	168	20.90	89	28	0.94	198	98	31	147.4	14.2	b/b
130	54	M	No	No	77	1.7	170	26.64	103	24	0.78	134	130	53.4	54.6	11.7	b/b

131	50	M	No	No	65	1.65	165	23.88	95	32	0.83	130	109	52	56.2	21.9	b/b
132	67	M	No	No	57	1.6	160	22.27	114	35	0.86	116	85	43	56	7.3	a/a
133	48	M	Yes	No	55	1.65	165	20.20	105	38	1.04	194	137	39.6	127	13.9	b/b
134	43	M	Yes	Yes	60	1.65	165	22.04	97	32	0.9	127	176	36	55.8	18.1	b/b
135	60	M	No	No	96	1.6	160	37.50	103	21	0.79	143	138	33	82.4	11.3	b/b
136	35	F	No	No	64	1.55	155	26.64	98	26	0.94	173	102	42.2	110.4	17.5	b/b
137	55	M	No	No	60	1.63	163	22.58	84	25	0.73	149	123	36.4	88	8.8	a/b
138	70	M	No	No	53	1.52	152	22.94	96	26	0.85	156	130	38.2	91.8	17.9	b/b
139	46	M	No	No	72	1.63	163	27.10	104	23	0.69	188	151	38	182	12.1	b/b
140	56	F	No	No	62	1.68	168	21.97	102	21	0.82	141	168	38	110	18.6	b/b
141	65	M	Yes	Yes	69	1.71	171	23.60	89	24	0.71	191	201	35	160	14.8	b/b
142	46	F	No	No	70	1.61	161	27.01	99	27	0.94	188	151	38	182	21.3	b/b
143	59	M	No	Yes	73	1.72	172	24.68	106	29	0.86	211	186	38	157	11.7	b/b
144	35	F	No	No	64	1.55	155	26.64	84	35	0.84	173	102	42.2	110.4	16.8	b/b
145	29	M	Yes	Yes	65	1.69	169	22.76	93	34	0.82	156	182	35	84.6	14.9	b/b
146	50	M	No	No	68	1.78	178	21.46	107	37	0.79	156	173	43	78.4	16.2	b/b
147	47	M	No	No	70	1.62	162	26.67	105	25	0.65	165	168	51	80.4	13.8	b/b
148	50	M	No	No	90	1.71	171	30.78	88	23	0.73	174	142	34	111.6	17.4	b/b
149	34	M	Yes	Yes	76	1.76	176	24.54	89	32	0.76	125	163	55	37.4	13.8	b/b
150	50	M	No	No	62	1.67	167	22.23	95	31	0.75	141	149	38	73.2	21.2	b/b

Table 2

Master chart for controls

Sl. No	Age	Sex	smoking	Alcoholism	Wt. (Kg)	Height(m)	BMI	plasma glucose fasting (mg/dl)	serum urea (mg/dl)	serum creatinine (mg/dl)	CHOL (mg/dl)	TGL (mg/dl)	HDL (mg/dl)	LDL (mg/dl)	SERUM TOTAL NITRITES(µmol/L)	eNOS Intron4 genotype
1	45	M	No	No	70	1.6	27.04	98	24	1.1	110	136	51.4	31.4	17.2	b/b
2	74	F	No	No	50	1.5	22.22	89	27	1.06	149	189	33.5	77.7	18.5	b/b
3	50	M	No	No	60	1.55	25	101	37	0.96	134	151	34.6	69.2	17	b/b
4	55	M	No	No	70	1.64	26.92	87	26	0.95	150	278	31.9	62.5	73	b/b
5	88	M	No	No	65	1.59	25.79	98	28	0.86	149	278	31.9	61.5	15.5	a/b
6	50	M	No	No	68	1.58	27.24	94	24	0.79	145	120	57.5	63.5	34.8	b/b
7	74	M	No	No	60	1.6	23.44	100	32	0.69	121	125	42.8	53.2	14.6	a/b
8	60	F	No	No	70	1.55	29.14	104	35	0.87	129	109	55	52.2	38.1	b/b
9	80	M	No	No	50	1.5	22.22	87	27	0.86	180	243	38	93.4	23.3	b/b
10	52	M	yes	yes	70	1.6	27.34	82	24	0.76	149	96	47.6	82.2	12.2	b/b
11	65	M	No	No	80	1.7	27.68	95	27	0.59	136	230	45	45	14.2	b/b
12	61	M	No	No	60	1.58	24.03	94	20	0.96	149	248	48	51.4	21.1	b/b
13	65	M	No	No	55	1.54	23.19	85	24	0.58	152	52	75	66.6	13.5	b/b
14	60	M	No	No	85	1.75	27.76	82	26	0.96	110	142	38.6	43	8	a/b
15	72	M	No	No	75	1.7	25.95	94	32	1	164	107	55.3	87.3	17.3	b/b
16	70	M	No	No	85	1.78	26.83	102	31	0.97	140	203	38.1	61.3	14.5	b/b
17	69	M	No	No	60	1.7	20.76	95	21	0.9	161	200	41.2	79.8	14.6	b/b
18	50	F	No	No	75	1.55	31.22	81	25	0.8	192	121	51	116.8	23.5	b/b
19	50	M	No	No	80	1.5	35.56	104	27	0.84	156	72	58	83.6	11.5	a/b
20	60	M	No	No	65	1.65	23.88	92	31	0.71	148	75	39	94	23.6	b/b
21	86	M	No	No	60	1.7	20.76	96	20	0.87	138	81	42	79.8	15.1	b/b
22	48	M	yes	yes	70	1.6	27.34	93	24	0.6	230	281	82	91.8	28.5	b/b
23	50	M	No	No	55	1.5	24.44	84	31	0.98	201	244	62.4	89.8	21.1	b/b
24	40	M	No	No	72	1.6	28.13	89	34	0.98	157	303	43	53.4	21.8	b/b
25	60	M	No	No	61	1.63	22.96	95	25	0.7	147	131	72	48.8	31.6	b/b
26	70	F	No	No	65	1.58	26.04	91	27	0.86	132	145	53	50	9.6	a/b
27	50	M	No	No	80	1.65	29.38	92	32	0.9	159	148	45	84.4	24.9	b/b
28	50	F	No	No	68	1.58	27.24	85	27	0.89	144	128	53	65.4	17.5	b/b
29	60	M	yes	yes	65	1.53	27.77	88	31	0.84	224	336	48.2	108.6	21.4	b/b
30	67	M	No	No	90	1.8	27.78	84	28	0.82	132	128	54	52.4	19.8	b/b
31	48	M	No	No	72	1.71	24.62	97	28	0.95	152	52	75	66.6	14.1	b/b
32	40	M	No	No	50	1.65	18.37	84	25	0.9	160	173	42.3	83.1	11.1	a/b
33	45	M	yes	yes	45	1.5	20	103	36	0.85	149	189	53.5	57.7	14.7	b/b
34	60	M	yes	yes	65	1.61	25.08	92	26	0.91	322	300	74.7	187.3	18.4	b/b
35	75	M	No	No	50	1.5	22.22	87	28	1.03	208	295	41.5	107.5	21.7	b/b
36	41	M	No	No	65	1.5	28.89	81	39	1.1	174	116	51	99.8	18	b/b
37	52	M	yes	yes	55	1.62	20.96	95	35	0.99	221	293	45.8	116.6	17.4	b/b
38	59	M	No	No	55	1.7	19.03	82	36	0.79	310	370	60	176	10.5	a/b
39	54	M	No	No	55	1.55	22.89	96	39	1.03	215	294	50.2	106	23.2	b/b
40	60	M	yes	No	50	1.5	22.22	91	25	0.98	219	152	73.9	114.7	8.2	a/a
41	50	M	yes	yes	60	1.57	24.34	104	31	0.7	194	155	47	116	21.5	b/b
42	60	M	yes	yes	42	1.5	18.67	84	36	0.86	170	101	45.8	104	19.6	b/b
43	55	M	No	No	55	1.55	22.89	96	23	0.8	149	83	42.6	89.8	10.1	a/b
44	65	M	No	No	55	1.54	23.19	101	32	0.86	193	128	54	113.4	18.2	b/b
45	60	F	No	No	50	1.55	20.81	104	39	0.87	368	244	75.7	243.5	16.4	b/b
46	60	M	No	No	74	1.6	28.91	85	24	0.78	356	273	76.6	224.8	17.2	b/b
47	40	M	No	No	70	1.6	27.34	82	26	0.59	154	119	61	69.2	14.3	b/b
48	44	M	No	No	75	1.7	25.95	95	27	0.68	210	271	47.5	108.3	18.7	b/b
49	55	F	No	No	68	1.65	24.98	89	25	0.87	210	151	55.1	124.7	16	b/b
50	60	M	yes	No	65	1.6	25.39	104	28	0.96	105	95	48	38	9.2	a/b

51	55	M	yes	yes	50	1.6	19.53	83	31	1.1	147	132	51	69.6	21	b/b
52	45	M	yes	yes	65	1.58	26.04	102	28	0.71	143	138	41	74.4	17.2	b/b
53	40	M	yes	No	68	1.6	26.56	88	29	0.7	151	132	45.2	79.4	10.9	a/b
54	48	M	No	No	53	1.58	21.23	94	37	0.75	153	130	46.7	80.3	18.1	b/b
55	36	M	No	No	65	1.65	23.88	99	25	0.79	136	125	38.5	72.5	15.7	b/b
56	40	M	No	No	59	1.64	21.94	92	31	0.84	153	80	53	84	17.5	b/b
57	42	M	No	No	58	1.58	23.23	89	27	0.95	161	137	56	77.6	13.1	a/b
58	49	M	No	No	53	1.58	21.23	86	28	0.58	281	217	81.5	156.1	17.2	b/b
59	32	M	No	No	55	1.54	23.19	83	37	0.63	210	280	45.1	108.9	8.9	a/a
60	48	M	yes	No	75	1.6	29.3	89	27	0.94	162	159	56	74.2	24.7	b/b
61	49	M	No	No	70	1.65	25.71	105	32	0.94	188	112	50.4	115.2	21.5	b/b
62	52	M	No	No	64	1.65	23.51	102	26	0.78	120	134	34	59.2	16.8	b/b
63	55	M	No	No	60	1.63	22.58	84	25	0.83	149	123	36.4	88	18.6	b/b
64	70	M	No	No	53	1.52	22.94	96	37	0.86	156	130	38.2	91.8	17.6	b/b
65	53	M	No	No	56	1.63	21.08	92	40	1.04	138	156	26.4	80.4	12.1	a/b
66	53	M	Yes	Yes	55	1.62	20.96	102	32	0.9	143	151	33.6	79.2	18.6	b/b
67	28	M	No	No	52	1.56	21.37	108	31	0.79	138	162	45.1	60.5	19.3	b/b
68	63	M	No	No	54	1.66	19.60	105	35	0.94	162	92	38	105.6	13.2	b/b
69	58	M	No	No	64	1.55	26.64	100	37	0.79	189	171	24	130.8	10.8	a/b
70	25	M	Yes	No	55	1.58	22.03	98	24	0.94	166	154	39	96.2	17.8	b/b
71	51	M	No	No	63	1.76	20.34	93	25	0.73	145	167	32	79.6	19.7	b/b
72	48	M	Yes	Yes	58	1.67	20.80	96	37	0.85	175	166	28	113.8	23.8	b/b
73	45	M	Yes	Yes	72	1.76	23.24	92	25	0.69	126	152	38.3	57.3	9.8	a/b
74	58	M	No	No	49	1.48	22.37	102	31	0.82	125	136	43	54.8	29.7	b/b
75	35	M	No	No	72	1.64	26.77	108	38	0.71	181	138	37.3	116.1	18.6	b/b
76	50	M	No	No	51	1.55	21.23	103	37	0.65	175	192	41	95.6	19.5	b/b
77	51	M	No	No	57	1.56	23.42	100	36	0.87	151	148	45	76.4	21.1	b/b
78	54	F	No	No	75	1.51	32.89	98	26	1.09	185	143	37	119.4	21.4	b/b
79	60	F	No	No	75	1.53	32.04	102	29	0.67	146	153	36	79.4	23.6	b/b
80	50	M	Yes	No	65	1.7	22.49	97	34	0.87	148	182	38	73.6	12.1	a/b
81	52	M	Yes	No	80	1.7	27.68	85	26	0.95	168	174	28	105.2	24.5	b/b
82	29	M	Yes	Yes	65	1.69	22.76	93	36	0.86	156	182	35	84.6	19.6	b/b
83	50	M	No	No	68	1.78	21.46	100	38	1.04	156	173	43	78.4	16.8	b/b
84	47	M	No	No	70	1.62	26.67	102	25	0.95	165	168	51	80.4	17.8	b/b
85	50	M	No	No	90	1.71	30.78	88	29	0.84	174	142	34	111.6	17.5	b/b
86	34	M	Yes	Yes	76	1.76	24.54	89	29	0.96	125	163	55	37.4	10.8	a/b
87	50	M	No	No	62	1.67	22.23	92	34	0.9	141	149	38	73.2	18.7	b/b
88	33	M	Yes	Yes	68	1.63	25.59	95	32	0.79	117	199	35.4	41.8	11.9	a/a
89	48	M	Yes	No	55	1.65	20.20	88	31	0.75	194	137	39.6	127	18.9	b/b
89	43	M	Yes	Yes	60	1.65	22.04	97	25	0.73	127	176	36	55.8	18.8	b/b
90	60	M	No	No	96	1.6	37.50	102	25	0.94	143	138	33	82.4	22.9	b/b
91	35	F	No	No	64	1.55	26.64	103	31	0.9	173	102	42.2	110.4	21.9	b/b
92	55	M	No	No	54	1.53	23.07	93	26	0.7	146	271	34.3	57.5	19.7	b/b
93	40	M	No	No	67	1.71	22.91	98	29	1.05	212	194	38	135.2	22.6	b/b
94	47	M	Yes	No	73	1.63	27.48	95	34	0.69	206	153	41	134.4	12.1	a/b
95	56	M	No	No	75	1.61	28.93	95	31	1.1	147	113	33.5	90.9	11.9	a/b
96	63	M	No	No	67	1.72	22.65	103	25	0.8	114	201	45	28.8	16.8	b/b
97	52	M	No	No	75	1.71	25.65	86	35	0.76	123	197	52	31.6	18.7	b/b
98	59	M	No	No	62	1.68	21.97	94	37	0.74	149	187	28.1	83.5	17.9	b/b
99	52	M	No	No	64	1.65	23.51	102	26	0.93	120	134	34	59.2	19.7	b/b
100	55	M	No	No	60	1.63	22.58	84	27	0.8	149	123	36.4	88	12.6	a/b
101	70	M	No	No	53	1.52	22.94	96	36	0.96	156	130	38.2	91.8	23.6	b/b
102	46	M	No	No	72	1.63	27.10	89	33	0.84	188	151	38	182	8.7	a/a
103	56	F	No	No	62	1.68	21.97	102	26	0.63	141	168	38	110	13.8	b/b

104	65	M	Yes	Yes	69	1.71	23.60	83	28	0.82	191	201	35	160	9.7	b/b
105	46	F	No	No	70	1.61	27.01	102	22	0.91	188	151	38	182	17.9	b/b
106	59	M	No	Yes	73	1.72	24.68	103	32	0.97	211	186	38	157	30.6	b/b
107	35	F	No	No	64	1.55	26.64	99	28	0.95	173	102	42.2	110.4	13.8	a/b
108	29	M	Yes	Yes	65	1.69	22.76	93	31	0.86	156	182	35	84.6	17.8	b/b
109	50	M	No	No	68	1.78	21.46	105	24	1.04	156	173	43	78.4	15.8	b/b
110	47	M	No	No	70	1.62	26.67	97	35	0.89	165	168	51	80.4	17.7	b/b
111	50	M	No	No	90	1.71	30.78	88	37	0.86	174	142	34	111.6	18.9	b/b
112	34	M	Yes	Yes	76	1.76	24.54	89	41	0.7	125	163	55	37.4	12.9	b/b
113	50	M	No	No	62	1.67	22.23	95	25	0.71	141	149	38	73.2	23.6	b/b
114	33	M	Yes	Yes	68	1.63	25.59	104	36	0.84	117	199	35.4	41.8	19.7	b/b
115	45	M	No	No	53	1.52	22.94	96	34	1.1	156	130	38.2	91.8	17.6	b/b
116	64	M	No	No	56	1.63	21.08	92	37	1	138	156	26.4	80.4	12.1	a/b
117	54	M	Yes	Yes	55	1.62	20.96	102	25	0.98	143	151	33.6	79.2	18.6	b/b
118	38	M	No	No	52	1.56	21.37	108	37	0.9	138	162	45.1	60.5	19.3	b/b
119	42	M	No	No	54	1.66	19.60	105	34	0.87	162	92	38	105.6	13.2	b/b
120	59	M	No	No	64	1.55	26.64	100	26	0.85	189	171	24	130.8	10.8	a/b
121	37	M	Yes	No	55	1.58	22.03	98	28	0.84	166	154	39	96.2	17.8	b/b
122	47	M	No	No	63	1.76	20.34	93	34	0.86	145	167	32	79.6	19.7	b/b
123	52	M	Yes	Yes	58	1.67	20.80	96	39	0.75	175	166	28	113.8	23.8	b/b
124	48	M	Yes	Yes	72	1.76	23.24	92	34	0.69	126	152	38.3	57.3	9.8	a/b
125	39	M	No	No	49	1.48	22.37	102	25	1.07	125	136	43	54.8	29.7	b/b
126	44	M	No	No	72	1.64	26.77	108	25	0.59	181	138	37.3	116.1	18.6	b/b
127	53	M	No	No	51	1.55	21.23	103	26	0.68	175	192	41	95.6	19.5	b/b
128	47	M	No	No	70	1.62	26.67	102	23	0.87	165	168	51	80.4	17.8	b/b
129	50	M	No	No	90	1.71	30.78	88	21	0.96	174	142	34	111.6	17.5	b/b
130	34	M	Yes	Yes	76	1.76	24.54	89	22	0.93	125	163	55	37.4	10.8	a/b

(11)

INSTITUTIONAL ETHICAL COMMITTEE
MADRAS MEDICAL COLLEGE, CHENNAI-600 003

Telephone 25363970
Fax 044 2535115

L.Dis.No.14597/ME5/Ethics Dean/MMC/2010 Dated : 12.05.2010

Title of the work : "Endothelial Nitric Oxide Synthase Gene Polymorphism in Patients with Hypertension in South Indian Population".

Principal Investigator : Dr. G. Sasirekha.
Designation : PG in MD Biochemistry.
Department : MMC, Ch-3.

The request for an approval from the Institutional Ethical Committee (IEC) was considered on the IEC meeting held on 12th May 2010 at 2.p.m in Pharmacology Seminar Hall, Madras Medical College, Chennai -3

The members of the Committee, the Secretary and the Chairman are pleased to approve the proposed work mentioned above, submitted by the principal investigator.

The Principal investigator and their team are directed to adhere to the guidelines given below:

1. You should get detailed informed consent from the patients/participants and maintain confidentiality.
2. You should carry out the work without detrimental to regular activities as well as without extra expenditure to the Institution or Government.
3. You should inform the IEC in case of changes in study procedure, site investigator investigation or guide or any other changes.
4. You should not deviate form the area of the work for which you applied for ethical clearance.
5. You should inform the IEC immediately, in case of any adverse events or serious adverse reactions.
6. You should abide to the rules and regulation of the institution(s).
7. You should complete the work within the specified period and if any extension of time is required, you should apply for permission again and do the work.
8. You should submit the summary of the work to the ethical committee on completion of the work.
9. You should not claim funds from the Institution while doing the work or on completion.
10. You should understand that the members of IEC have the right to monitor the work with prior intimation.

SECRETARY
IEC, MMC,CHENNAI

CHAIRMAN
IEC,MMC, CHENNAI

DEAN
MADRAS MEDICAL COLLEGE,
CHENNAI -3